REMARKS

Claims 1 to 11 and 29 to 32 are cancelled without prejudice. Claims 12 to 28 and 33 to 34 are pending in the application. Claims 12, 13, 20, 21 and 24 to 26 are amended. Claims 13, 20 and 24 to 26 are amended to correct grammatical errors. Claims 12, 20 and 21 are further amended as indicated below. Claims 33 and 34 are new and incorporate subject matter from claims 10 and 11.

Claims 12 to 28 are objected to for containing non-elected subject matter which must be removed from the claims. Additionally, claim 12 should be written to incorporate the subject matter of the non-elected claim from which it depends. In response, claim 12 has been amended to independent form and to remove non-elected subject matter. It is believed that these amendments render the objections moot and withdrawal is respectfully requested.

Claims 12 to 28 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention. The Examiner states that claim 12 should define a nucleic acid sequence by a single nucleic acid sequence as defined in a single SEQ ID NO., or by the specific amino acid sequence it encodes, e.g. SEQ ID NO: 14. Claim 12 should also recite that the nucleic acid sequence is isolated and/or purified. As requested by the Examiner, claim 12 has been amended to recite the elected SEQ ID NO: 14 and to recite that the nucleic acid sequence is isolated and/or purified. Withdrawal of the rejection is respectfully requested.

The Examiner states that claim 16 should recite the appropriate deposit identification numbers for the host cells, otherwise it is vague and indefinite what is encompassed by the names recited therein. The Examiner states that the mere recitation of a name, i.e., JM101, to describe the invention is not sufficient to satisfy the statute's requirement of adequately describing and setting forth the inventive concept. Applicant respectfully disagrees. The host cells recited in claim 16 are well-known in the art and are commercially available. In support, a reference for each cell line is enclosed herewith as follows:

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Stratagene catalogue for NM522; Sigma technical bulletin for BL21; Invitrogen catalogue pages for DH5α; Stratagene catalogue pages for JM101; for JM105, de Taxis du Poët, Applied and Environmental Microbiology (1987) 53:1548-1555; and for N99CI+, Hinton *et al.*, The Journal of Biological Chemistry (1985) 260:12851-12857. Applicant respectfully submits that the cells recited in claim 16 are therefore clearly and distinctly defined by name and that the claim is in compliance with 35 U.S.C. 112, second paragraph.

The Examiner states that claim 20 is vague and indefinite due to the term "inducer". Claim 20 has been amended for clarity. Applicant notes that the term "suitable conditions" has been removed from the claim. In addition, Applicant respectfully submits that inducers are well-known in the art and the specification describes several alternatives, for example, on page 10, lines 23 to 24. Applicant respectfully submits therefore that the claim is in compliance with 35 U.S.C. 112, second paragraph.

The Examiner states that claim 21 is vague and indefinite for the condition "temperature", and asks what is encompassed by this term. Claim 21 has been amended to indicate that the temperature is "suitable for inducing expression of the vector". Applicant respectfully submits that the claim is therefore in compliance with 35 U.S.C. 112, second paragraph.

The amendments to the claims and/or arguments as presented above are believed to overcome all of the Examiner's rejections under 35 U.S.C. 112, second paragraph.

Claims 12 to 28 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for "an isolated nucleic acid sequence which encodes an amino acid sequence as set forth in SEQ ID NO: 14 and expression vectors, host cells and methods of recombinant production comprising/using this sequence, does not reasonably provide enablement for a nucleic acid sequence encoding the fusion protein of claim 6...". The Examiner also states that, while the specification states that substitutions, additions, or deletions may be made to the

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defined sequences, the specification provides no guidance as to what nucleic acid may be changed without causing a detrimental effect to the protein to be produced.

Independent claim 12 has been amended to specify that the fusion carrier protein encoded by the nucleic acid sequence consists of SEQ ID NO: 14. It is believed therefore that the rejections are rendered moot. Applicant respectfully submits that the claims comply with the first paragraph of 35 U.S.C. 112, and withdrawal of the rejection is respectfully requested.

Claims 12 to 28 are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. The written description in this case only sets forth an isolated nucleic acid sequence encoding an amino acid sequence as set forth in SEQ ID NO: 14 and therefore the written description is not commensurate in scope with the claims.

Independent claim 12 has been amended to specify that the fusion carrier protein encoded by the nucleic acid sequence consists of SEQ ID NO: 14. It is believed that the rejection is therefore rendered moot and withdrawal is respectfully requested.

Claim 16 is rejected under 35 U.S.C. 112, first paragraph as failing to comply with the enablement requirement. The Examiner states that the specification lacks complete deposit information for the deposit of host cells DH5α, BL21, JM101 or JM105 or NM522 or N99CI+. The Examiner states that it is not clear that the properties of these host cells are known and publicly available or can be reproducibly isolated from nature without undue experimentation. As discussed above, the host cells recited in claim 16 are well-known in the art and are publicly and commercially available, as evidenced by the attached references which are detailed above. Applicant respectfully submits therefore that a biological deposit is not required and withdrawal of the rejection is earnestly solicited.

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Claims 12 to 17 and 19 to 22 are rejected under 35 U.S.C 102(b) as being anticipated by Kovacevic *et al.* (US 4,977,089). Kovacevic *et al.* describe the full length S.aureus nuclease protein. The claims have been amended to recite the fusion carrier protein consisting of an amino acid sequence as set forth in SEQ ID NO: 14. Applicant notes that the full length S.aureus sequence is not encompassed within the scope of the amended claims. Kovacevic *et al.* do not teach SEQ ID NO: 14, as claimed herein. Applicant respectfully submits that the claims are not anticipated by Kovacevic *et al.* and withdrawal of the rejection is earnestly solicited.

Claims 12 to 28 are rejected under 35 U.S.C. 102(b) as being anticipated by Murray *et al.* (US 6,365,347). Murray *et al.* also describe the full length S.aureus nuclease protein. The claims have been amended to recite the fusion carrier protein consisting of an amino acid sequence as set forth in SEQ ID NO: 14. The claim does not use the open language, thus the use of the full length S.aureus sequence is not encompassed within the scope of the amended claims. Therefore, the claims are not anticipated by Murray *et al.* and withdrawal of the rejection is earnestly solicited.

Claims 23 to 28 rejected under 35 U.S.C. 103(a) as being unpatentable over Kovacevic *et al.* (US 4,977,089). The claims have been amended to recite the fusion carrier protein consisting of an amino acid sequence as set forth in SEQ ID NO: 14. Kovacevic *at al.* do not teach or suggest the use of the fragment SEQ ID NO: 14 as a fusion carrier protein. Therefore it is respectfully submitted that the claimed invention is not obvious in view of the cited reference and withdrawal of the rejection is earnestly solicited.

Applicant submits that no new matter has been added by the present amendments.

Therefore, it is submitted that the claims are in condition for allowance, and reconsideration of the Examiner's rejections is respectfully requested. Allowance of claims 12 to 28 and 33 to 34 at an early date is solicited.

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In the event that there are any questions concerning this amendment or the application in general, the Examiner is respectfully urged to telephone the undersigned so that prosecution of this application may be expedited.

No additional fees are believed to be necessitated by this amendment. However, should this be in error, authorization is hereby given to charge Deposit Account No. 19-5113 for any underpayment or to credit any overpayment.

Respectfully submitted,

Date: October 26, 2007

By: 
Emma Saffman, Reg. No. 56,699
Agent of record

OGILVY RENAULT
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Tel: (514) 847-4337

Enc.: Stratagene catalogues; Sigma technical bulletin; Invitrogen catalogue; de Taxis du Poët, Applied and Environmental Microbiology (1987); Hinton *et al.*, The Journal of Biological Chemistry (1985)

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October 26, 2007
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NM522 Competent Cells

Catalog #200233

MATERIALS PROVIDED

Materials provided	Quantity	Efficiency (cfu/µg of pUC18 DNA)
NM522 competent cells (red-orange tubes)	5 × 0.2 ml	≥1 × 10^8
pUC18 control plasmid (0.1 ng/µl in TE buffer)	10 µl	—
β-Mercaptoethanol (1.42 M)	25 µl	—

Storage: Competent cells must be placed immediately at the bottom of a –80°C freezer directly from the dry ice shipping container. Do not store the cells in liquid nitrogen.

QUALITY CONTROL TESTING

Transformations are performed both with and without plasmid DNA using 100-µl aliquots of cells and 100 pg of pUC18 control plasmid following the protocol outlined below. Following transformation, 5-µl samples of the culture are plated in duplicate on LB agar plates with 100 µg/ml of ampicillin. The plates are incubated at 37°C overnight and the efficiency is calculated based on the average number of colonies per plate.

BACKGROUND

NM522 is a recombination-proficient and *Eco*K restriction-deficient (RecA$^-$, HsdR$^-$) host strain.

NM522 Genotype: *supE thi-1* Δ(*lac-proAB*) Δ(*mcrB-hsdSM*)*5* ($r_K^- m_K^+$) [F′ *proAB lacI*^{q}*ZΔM15*]. (Genes listed signify mutant alleles. Genes on the F′ episome, however, are wild-type unless indicated otherwise.)

NM522 cells contain the *lacI*^{q}*ZΔM15* gene on the F′ episome, allowing blue-white screening for recombinant plasmids.

TRANSFORMATION PROTOCOL

1. Pre-chill two 14-ml BD Falcon polypropylene round-bottom tubes on ice. (One tube is for the experimental transformation and one tube is for the pUC18 control.) Preheat SOC medium[§] to 42°C.
2. Thaw the competent cells on ice. When thawed, gently mix and aliquot 100 µl of cells into each of the two pre-chilled tubes.
3. Add 1.7 µl of β-mercaptoethanol provided with this kit to each aliquot of cells.
4. Swirl the contents of the tubes gently. Incubate the cells on ice for 10 minutes, swirling gently every 2 minutes.
5. Add 0.1–50 ng of the experimental DNA (see *Quantity and Volume of DNA*, reverse page, for guidelines) to one aliquot of cells and add 1 µl of the pUC18 control DNA to the other aliquot. Swirl the tubes gently.
6. Incubate the tubes on ice for 30 minutes.
7. Heat-pulse the tubes in a 42°C water bath for 45 seconds. The duration of the heat pulse is **critical** for maximum efficiency.
8. Incubate the tubes on ice for 2 minutes.
9. Add 0.9 ml of preheated SOC medium and incubate the tubes at 37°C for 1 hour with shaking at 225–250 rpm.
10. Plate ≤200 µl of the transformation mixture on LB agar plates containing the appropriate antibiotic (and containing IPTG and X-gal if color screening is desired).[§] For the pUC18 control transformation, plate 5 µl of the transformation on LB–ampicillin agar plates.[§]

 Notes *Cells may be concentrated by centrifuging at 1000 rpm for 10 minutes. Resuspend the pellet in 200 µl of SOC medium.*

 If plating <100 µl of cells, pipet the cells into a 200 µl pool of SOC medium and then spread the mixture with a sterile spreader. If plating ≥100 µl, the cells can be spread on the plates directly. Tilt and tap the spreader to remove the last drop of cells.

 Some β-galactosidase fusion proteins are toxic to the host bacteria. If an insert is suspected to be toxic, plate the cells on media without X-gal and IPTG. Color screening will be eliminated, but lower levels of the potentially toxic protein will be expressed.
11. Incubate the plates at 37°C overnight (at least 17 hours for blue-white color screening). Colonies containing plasmids with inserts will remain white, while colonies containing plasmids without inserts will be blue. The blue color can be enhanced by incubating the plates for two hours at 4°C following the overnight incubation at 37°C.
12. For the pUC18 control, expect 50 colonies (≥1 × 10^8 cfu/µg pUC18 DNA). For the experimental DNA, the number of colonies will vary according to the size and form of the transforming DNA, with larger and non-supercoiled DNA producing fewer colonies.

[§]See *Preparation of Media and Reagents*.

Transformation Guidelines and Troubleshooting

Storage Conditions: Competent and supercompetent cells are very sensitive to even small variations in temperature and must be stored at the bottom of a –80°C freezer. Transferring tubes from one freezer to another may result in a loss of efficiency.

Use of 14-ml BD Falcon polypropylene round-bottom tubes: It is important that 14-ml BD Falcon polypropylene round-bottom tubes (BD Biosciences Catalog #352059) are used for the transformation protocol, since other tubes may be degraded by β-mercaptoethanol. In addition, the duration of the heat-pulse is critical and has been optimized for these tubes.

Aliquoting Cells: Keep the cells on ice at all times during aliquoting. It is essential that the polypropylene tubes are placed on ice before the cells are thawed and that the cells are aliquoted directly into pre-chilled tubes. It is also important to use the volume of cells indicated in step 2 of the *Transformation Protocol*. Decreasing the volume will result in lower efficiencies.

Use of β-Mercaptoethanol (β-ME): β-ME has been shown to increase transformation efficiency. The β-ME provided is diluted and ready to use. A fresh 1:10 dilution (from a 14.2 M stock) may be used; however, Stratagene cannot guarantee results with β-ME from other sources.

Quantity and Volume of DNA: The greatest efficiency is obtained from the transformation of 1 µl of 0.1 ng/µl supercoiled pUC18 DNA per reaction. A greater number of colonies may be obtained by transforming up to 50 ng DNA, although the resulting efficiency (cfu/µg DNA) may be lower. The volume of the DNA solution added to the reaction may be increased to up to 10% of the reaction volume, but the transformation efficiency may be reduced.

Heat Pulse Duration: Optimal transformation efficiency is observed when cells are heat-pulsed at 42°C for 45–50 seconds. Efficiency decreases sharply when cells are heat-pulsed for <45 seconds or for >60 seconds.

Blue-White Color Screening: Blue-white color screening for recombinant plasmids is available when transforming host strains that contain the *lacI*^{q}ZΔ*M15* gene on the F′ episome with a plasmid that provides α-complementation (e.g. Stratagene's pBluescript® II vector). When performing blue-white color screening, incubate the LB agar plates containing IPTG and X-gal at 37°C for at least 17 hours to allow color development. The blue color can be enhanced by subsequent incubation of the plates for two hours at 4°C.

Preparation of Media and Reagents

SOB Medium (per Liter) 20.0 g of tryptone 5.0 g of yeast extract 0.5 g of NaCl Add deionized H_2O to a final volume of 1 liter Autoclave Add 10 ml of filter-sterilized 1 M $MgCl_2$ and 10 ml of filter-sterilized 1 M $MgSO_4$ prior to use	**SOC Medium (per 100 ml)** *Note This medium should be prepared immediately before use.* 2 ml of filter-sterilized 20% (w/v) glucose or 1 ml of filter-sterilized 2 M glucose SOB medium (autoclaved) to a final volume of 100 ml
LB Agar (per Liter) 10 g of NaCl 10 g of tryptone 5 g of yeast extract 20 g of agar Add deionized H_2O to a final volume of 1 liter Adjust pH to 7.0 with 5 N NaOH Autoclave	**LB–Ampicillin Agar (per Liter)** 1 liter of LB agar, autoclaved Cool to 55°C Add 10 ml of 10 mg/ml filter-sterilized ampicillin Pour into petri dishes (~25 ml/100-mm plate)

Preparation of Agar Plates for Blue-White Color Screening

To prepare plates for blue-white screening, prepare LB agar as indicated above. When adding the antibiotic, also add 5-bromo-4-chloro-3-inodlyl-β-D-galactopyranoside (X-gal) to a final concentration of 80 µg/ml [prepared in dimethylformamide (DMF)] and isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 20 mM (prepared in sterile dH_2O). Alternatively, 100 µl of 10 mM IPTG and 100 µl of 2% X-gal may be spread on solidified LB agar plates 30 minutes prior to plating the transformations. (For consistent color development across the plate, pipet the X-gal and the IPTG into a 100-µl pool of SOC medium and then spread the mixture across the plate. Do not mix the IPTG and the X-gal before pipetting them into the pool of SOC medium because these chemicals may precipitate.)

Limited Product Warranty

This warranty limits our liability to replacement of this product. No other warranties of any kind, express or implied, including without limitation, implied warranties of merchantability or fitness for a particular purpose, are provided by Stratagene. Stratagene shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

Endnotes

pBluescript® is a registered trademark of Stratagene in the United States.

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ProductInformation

BL21-T1^R Competent Cells, Uni-Pack

Product Number **B 2685**
Storage Temperature –70 °C

TECHNICAL BULLETIN

Product Description
BL21-T1^R competent *Escherichia coli* is an all-purpose expression strain suitable for the high level production of heterologous proteins directed by various expression vector systems (promoters such as *lac*, *trc*, *tac*, λPL, and *ara*D). BL21-T1^R is a B/r strain bacteria[1], which naturally lacks the *lon* protease[2] and is also deficient in the outer membrane protease *ompT*. The absence of proteolytic activity from these two proteases may reduce degradation of some heterologous proteins expressed in the strain. In addition, the *ton*A genotype confers resistance to the lytic bacteriophages T1 and T5 for protection of clonal stocks.[3]

Sigma's BL21-T1^R competent *E. coli* cells are grown and made chemically competent using an optimized procedure specific to the strain, followed by strain verification and efficiency testing. The cells are provided in frozen 50 µl aliquots for convenience. Each aliquot can be used for a single transformation. The cells have a transformation efficiency of ≥3 x 10^8 cfu/µg when transformed with non-saturating amounts of pUC19 plasmid DNA.

Genotype of BL21-T1^R:
F$^-$ *ompT hsdS$_B$*($r_B^- m_B^-$) *gal dcm ton*A

Components
BL21-T1^R Competent Cells are packaged with sufficient reagents for 10 transformation reactions.

- BL21-T1^R Competent Cells, 10 X 50 µl, Product No. B 2810
- pUC19 Control DNA, 10 ng/µl, 10 µl, Product No. D 2567

Reagents and Equipment Required but Not Provided
(Sigma Product Numbers have been given where appropriate)

- Shaker Incubator (37 °C)
- Cabinet incubator (37 °C)
- Heated water bath (42 °C)
- SOC Medium, Product No. S 1797
- LB Agar EZMix™, Product No. L 7533
- LB Broth EZMix™, Product No. L 7658
- Appropriate selection antibiotic
- 15 ml polypropylene culture tubes (sterile)
- Culture dishes, Product No. C 6796
- Sterile Spreaders, Product No. Z37,677-9

Precautions and Disclaimer
Sigma BL21-T1^R Competent Cells are for laboratory use only, not for drug, household, or other uses. Consult the MSDS for information regarding hazards and safe handling procedures.

Storage/Stability
All components are stable for at least six months after receipt when properly stored at –70 °C.

Handling Tips:

1. Verify that the cells are still frozen and dry ice is still present in the shipping container upon receipt.
2. Handle the tubes as little as possible to prevent accidental warming of the cells. Keep cells on ice at all times.
3. When mixing the cells, gently swirl or tap the reaction tube. Do not mix by pipetting or vortexing.
4. Cells can be refrozen on dry ice and returned to –70 °C, however transformation efficiency will decrease significantly with each freeze-thaw cycle.

Procedure
Before Starting:

1. Prepare LB agar plates with the appropriate antibiotic for selection of colonies that contain plasmid DNA, and warm to 37 °C.

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2. Heat a water bath to 42 °C.

3. Warm SOC medium to room temperature (20-25 °C).

I. Standard Transformation Protocol In Brief

Note: See following section for detailed protocol

1. Thaw the required number of tubes containing cells on wet ice.

2. Add 1-50 ng of DNA (or 1 µl of control DNA) to cells and gently tap the tube to mix.

3. Place cells on wet ice for 30 minutes.

4. Heat shock cells by incubating tubes in a 42 °C water bath for **exactly** 45 seconds.

5. Return the cells to ice for 2 minutes.

6. Add 450 µl of SOC, at room temperature, to each transformation reaction.

7. Shake at 225 rpm at 37 °C for 1 hour.

8. Use a sterile spreader to evenly distribute transformation reaction over LB agar plates containing the appropriate selection antibiotic.

9. Incubate plates overnight at 37 °C.

II. Detailed Standard Transformation Protocol

Note: DNA in ligation reactions can be added directly to Sigma's competent cells. Inactivation of the ligase is not required prior to transformation. No more than 50 ng of ligation reaction should be used in a 50 µl transformation reaction.

Plasmid DNA isolated using miniprep procedures is typically satisfactory. To achieve maximum efficiency, the sample DNA should be free of phenol, ethanol, salts, protein, and detergents, and be dissolved in TE buffer or water.

1. Remove the required tubes of cells from the –70 °C freezer, including one extra for the control DNA if desired. Place tubes immediately on wet ice so that only the cap is visible above the ice. Allow the cells to thaw on ice for approximately 5 minutes.

2. Visually examine cells to ensure they are thawed, and gently tap the vial several times to resuspend cells.

3. (Optional) Add 1 µl pUC19 Control DNA (10 ng) to one tube of cells. Mix gently by tapping the tube. Return the cells to the ice.

4. Add 1 ng to 50 ng ligation reaction or purified plasmid DNA directly to cells. Mix as in Step 3.

5. Incubate the cells on wet ice for 30 minutes.

6. Heat shock the cells by incubating tubes in a 42 °C water bath for **exactly** 45 seconds.

7. Immediately return the cells to ice for 2 minutes.

8. Add 450 µl of SOC medium, at room temperature, into each tube containing the cell/DNA mixture. Optimal recovery may be achieved by transferring the cells to a sterile 15 ml polypropylene culture tube. The cap should be loose to ensure sufficient air exchange and aeration of the culture.

9. Incubate cells at 37 °C with shaking (225-250 rpm) for 1 hour.

10. Transfer 10-100 µl of each transformed cell suspension onto LB agar plates containing selection antibiotic and evenly distribute using a sterile spreader. Plates should be pre-warmed to 37 °C for optimal transformation efficiency. When inoculating less than 25 µl of cell suspension, first pipette a drop of SOC onto the plate and add cell suspension to the SOC.

Note: The amount of transformation mixture to plate varies with the efficiency of both the ligation and the competent cells. When using the control DNA, add no more than 10 µl into a drop of SOC on an LB agar plate containing 100 µg/ml ampicillin or carbenicillin.

11. Incubate plates overnight at 37 °C.

References

1. Wood, W. B., Host specificity of DNA produced by Escherichia coli: bacterial mutations affecting the restriction and modification of DNA. J. Mol. Biol., **16**, 118-133 (1966).
2. Phillips, T.A., *et al.*, lon gene product of Escherichia coli is a heat-shock protein. J. Bacteriol., **159**, 283-287 (1984).
3. Braun, V., and Hantke, K., Bacterial receptors for phages and colicins as constituents of specific transport system, in *Receptors and Recognition*,

Series B, Vol. 3, J. L. Reissig (Ed.), pp. 101-130.
(Chapman & Hall, Ltd., London, 1977).

Troubleshooting Guide

Problem	Cause	Solution
No Colonies on plate	Cells thawed and re-frozen due to improper handling	Obtain new product. Temperature of ultracold freezer should be –70 °C or colder.
	Old or expired cells	Obtain new product.
	No plasmid DNA added	Ensure plasmid DNA was added to cell.
	Input plasmid DNA missing antibiotic resistance ORF and/or replication of origin	Review cloning strategy.
	Low ligation efficiency	Plate increased volume of transformation reaction.
	Too much, or wrong antibiotic used	Adjust antibiotic levels in plates, Test a plate by streaking with an antibiotic resistant culture.
	Low efficiency transformation	Check to make sure transformation protocol was followed exactly.
		Plate increased volume of transformation reaction.
		Check efficiency using included pUC19 control.
Too Many Colonies on Plate or a Lawn of Growth is Observed.	High efficiency transformation or high amounts of input DNA	Plate decreased volume of transformation reaction.
	Too little antibiotic	Adjust antibiotic levels in plates.
	Expired antibiotic	Use fresh antibiotic in plates.
	Contaminated SOC media	Obtain fresh medium and maintain sterile technique.
Unexpected Growth on Plate (i.e. fungal or mold)	Contaminated plates	Prepare and spread plates in laminar flow hood to reduce contamination.
	Contaminated SOC media	Obtain fresh medium and maintain sterile technique.

SU/JWM 09/04

Please visit www.sigma-aldrich.com for a complete listing of competent cells and other transformation products offered by Sigma-Aldrich.

EZMix is a trademark of Sigma-Aldrich Biotechnology LP.

Sigma brand products are sold through Sigma-Aldrich, Inc.
Sigma-Aldrich, Inc. warrants that its products conform to the information contained in this and other Sigma-Aldrich publications. Purchaser must determine the suitability of the product(s) for their particular use. Additional terms and conditions may apply. Please see reverse side of the invoice or packing slip.

Subcloning Efficiency™ DH5α™ Chemically Competent *E. coli*

Catalog no. 18265-017

General Information and Kit Contents

Shipping and Storage

The kit is shipped on dry ice. Upon receipt, store the kit at -70°C. Do not store in liquid nitrogen.

Kit Contents

The kit contains the following reagents.

Reagent	Composition	Amount
DH5α™ cells	--	4 x 500 µl
pUC19	100 pg/µl in 5 mM Tris-HCl, 0.5 mM EDTA, pH 8	20 µl (2 ng)

Genotype

F⁻ ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ⁻

Quality Control Procedure

Competent cells (100 µl) are transformed with 500 pg of supercoiled pUC19 plasmid. Transformed cultures are plated on LB plates containing 100 µg/ml ampicillin and the transformation efficiency is calculated. Test transformations are performed in duplicate. Transformation efficiency should be > 1 x 10^6 cfu/µg DNA.

Untransformed cells are tested for:

- Absence of contamination by plating on five different LB plates containing either 100 µg/ml ampicillin, 50 µg/ml kanamycin, 15 µg/ml tetracycline, 20 µg/ml chloramphenicol, or 100 µg/ml streptomycin
- Inhibited growth on nitrofurantoin (*rec*A)
- Lac⁻ and Gal⁺ phenotypes
- Absence of lambda phage contamination

Note: Saturating amounts of control pUC19 DNA (25 ng) generates > 1 × 10^4 transformants from a 50 µl reaction.

Patent Information

This product is covered by U.S. patent no. 4,981,797 and foreign equivalents.

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Overview

Applications

Subcloning Efficiency™ DH5α™ Chemically Competent *E. coli* are suitable for:

- Routine subcloning into plasmid vectors
- Blue/white screening of transformants on selective plates containing Bluo-gal or X-gal
- High-quality plasmid preparation
- Transformation of large plasmids (up to 30 kb)
- Transiently hosting M13mp cloning vectors (see page 5)

Subcloning Efficiency™ DH5α™ are **not** suitable for generation of cDNA libraries. We recommend Max Efficiency™ DH5α™-T1^R Chemically Competent *E. coli* (Catalog no. 12034-013) or UltraMax™ DH5α™-ft Chemically Competent *E. coli* (Catalog no. 10643013).

Important

DH5α™ *E. coli* **does not require** IPTG to induce expression from the *lac* promoter even though the strain expresses the Lac repressor. The copy number of most plasmids exceeds the repressor number in the cells. If you are concerned about obtaining maximal levels of expression, add IPTG to a final concentration of 1 mM.

If blue/white screening is required to select for transformants spread 40 µl of 40 mg/ml X-Gal in dimethylformamide on top of the agar. Let the X-Gal diffuse into the agar for at least 1 hour.

General Handling

Be extremely gentle when working with competent cells. Competent cells are highly sensitive to changes in temperature or mechanical lysis caused by pipetting. Transformation should be started immediately following the thawing of the cells on ice. **Mix by swirling or tapping the tube gently, not by pipetting or vortexing.**

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Transforming Chemically Competent Cells

Materials Supplied by the User

You will need the following items for transformation:

- 37°C shaking and non-shaking incubator
- 10 cm diameter LB agar plates with appropriate antibiotic (100 µg/ml ampicillin to select transformants containing pUC19 control DNA)
- LB, YT, or SOC Medium
- Dry ice and ethanol
- 37°C water bath

Before Starting

- Prepare a dry ice/ethanol bath and maintain at -70°C
- Equilibrate a water bath to 37°C
- Spread X-Gal onto LB agar plates with antibiotic, if desired
- Warm the medium and plates in a 37°C incubator for 30 minutes
- Obtain a test tube rack that will hold all transformation tubes so that they can all be put into a 37°C water bath at once.

Transformation Procedure

The instructions provided below are for general use. Plasmid DNA should be free of phenol, ethanol, protein, and detergents for maximum transformation efficiency.

1. Briefly centrifuge the ligation reaction and place on wet ice.
2. Remove one 500 µl tube of DH5α™ cells and thaw on wet ice.
3. Place the required number of sterile 1.5 ml microcentrifuge tubes on wet ice.
4. Gently mix cells with the pipette tip and aliquot 50 or 100 µl into each microcentrifuge tube.
5. Re-freeze any unused cells in the dry ice/ethanol bath for 5 minutes before returning the tube to the -70°C freezer. **Do not use liquid nitrogen.**
6. Pipet 1 to 5 µl (1-10 ng DNA) of each ligation reaction directly into the competent cells and mix by tapping gently. **Do not mix by pipetting up and down.** Store the remaining ligation reaction at -20°C.
7. (Optional) Pipet 5 µl (500 pg) pUC19 control DNA into 100 µl competent cells and mix as described in Step 6.
8. Incubate the vial on ice for 30 minutes.
9. Heat-shock for exactly 20 seconds in the 37°C water bath for 50 µl volume (45 seconds for 100 µl transformation). Do not mix or shake.
10. Remove vial from the 37°C bath and place on ice for 2 minutes.
11. Add 900 to 950 µl of pre-warmed medium of choice to each vial. Proceed to next page.

Continued on next page

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Transforming Chemically Competent Cells, Continued

Procedure, continued

12. Place the vial in a microcentrifuge rack on its side and secure with tape to avoid loss of the vial. Shake the vial at 37°C for exactly 1 hour at 225 rpm in a shaking incubator.
13. Spread 20 µl to 200 µl from each transformation vial on separate, labeled LB agar plates. We recommend that you plate two different volumes.

 Note: You may have to dilute cells 1:10 to obtain well-spaced colonies. Generally ligations are at least 10-fold lower efficiency.
14. (Optional) For cells transformed with pUC19 control DNA, plate 100 µl of the transformation reaction, then serially dilute the transformation reaction 1:10 and 1:100 and plate 100 µl of each dilution on plates containing 100 µg/ml ampicillin.
15. Store the remaining transformation reaction at +4°C and plate out the next day, if desired. If necessary, cells may be concentrated by centrifuging in a microcentrifuge (5 seconds) and resuspending them in 100 µl. Plate 1 to 90 µl.
16. Invert the plates and incubate at 37°C overnight.
17. Select colonies and analyze by plasmid isolation, PCR, or sequencing.

Calculation

Calculate the transformation efficiency as transformants per 1 µg of pUC19 plasmid DNA. Be sure to account for dilution or concentration of cells (DF = dilution factor).

$$\frac{\text{\# of colonies}}{\text{500 pg transformed pUC19 DNA}} \times \frac{10^6\ \text{pg}}{\mu\text{g}} \times \frac{\text{1000 µl total transformation volume}}{\text{100 µl plated}} \times \text{DF} = \frac{\text{\# transformants}}{\mu\text{g plasmid DNA}}$$

Expected transformation efficiency: >1 x 10^6 cfu/µg supercoiled plasmid.

Note

Transformation efficiencies for cDNA and ligation of inserts to vectors will be lower than for a supercoiled control plasmid such as pUC19.

- For cDNA, transformation efficiencies may be 10- to 100-fold lower.
- For ligation of inserts to vectors, transformation efficiencies may be 10-fold lower.

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Using DH5α™ as a Transient Host

Introduction

DH5α™ competent *E. coli* support replication of M13mp vectors, but it does not support plaque formation. Plating on a lawn of *E. coli* containing the F episome will allow plaque formation.

Before Starting

You will need the following reagents:

- Dry ice/ethanol bath
- **Log-phase** *E. coli* containing the F episome (*e.g.* DH5α-FT™, DH5αF′™, DH5αF′IQ™, JM101, or JM107)
- Liquid top agar containing 50 µg/ml Bluo-gal or X-gal and 1 mM IPTG
- LB plates (no antibiotic)

Procedure

A general procedure is provided below for your convenience; you may have to optimize the protocol for your particular circumstances.

1. Remove one 500 µl tube of DH5α™ cells and thaw on wet ice.
2. Place the required number of sterile 1.5 ml microcentrifuge tubes on ice.
3. Gently mix cells with the pipette tip and aliquot 50 µl into each microcentrifuge tube.
4. Re-freeze any unused cells in the dry ice/ethanol bath for 5 minutes before returning the tube to the -70°C freezer. **Do not use liquid nitrogen.**
5. Transform 10 pg (or 1-10 ng of a RF ligation) of replicative form (RF) M13mp into 50 µl of competent cells.
6. Incubate the vial on ice for 30 minutes.
7. Heat-shock for exactly 20 seconds in the 37°C water bath. Do not mix or shake.
8. Remove vial from the 37°C bath and place on ice. Note: Since selection by antibiotic resistance is not necessary for plaque formation, recovery in medium is not necessary.
9. Take the log-phase *E. coli* containing the F episome and add to the liquid top agar.
10. Add 30-50 µl of the transformation reaction from Step 8 to the top agar.
11. Mix and pour the top agar onto LB plates (no antibiotic).
12. After the plate has solidified, invert and incubate at 37°C overnight or until plaques form.

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Technical Service

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2. Follow instructions on the page and fill out all the required fields.
3. To request additional MSDSs, click the 'Add Another' button.
4. All requests will be faxed unless another method is selected.
5. When you are finished entering information, click the 'Submit' button. Your MSDS will be sent within 24 hours.

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Technical Service, Continued

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JM101 Competent Cells

Catalog #200234

MATERIALS PROVIDED

Materials provided	Quantity	Efficiency (cfu/µg of pUC18 DNA)
JM101 competent cells (yellow tubes)	5 × 0.2 ml	≥1 × 10^8
pUC18 control plasmid (0.1 ng/µl in TE buffer)	10 µl	—
β-Mercaptoethanol (1.42 M)	25 µl	—

Storage: Competent cells must be placed immediately at the bottom of a –80°C freezer directly from the dry ice shipping container. Do not store the cells in liquid nitrogen.

QUALITY CONTROL TESTING

Transformations are performed both with and without plasmid DNA using 100-µl aliquots of cells and 100 pg of pUC18 control plasmid following the protocol outlined below. Following transformation, 5-µl samples of the culture are plated in duplicate on LB agar plates with 100 µg/ml of ampicillin. The plates are incubated at 37°C overnight and the efficiency is calculated based on the average number of colonies per plate.

BACKGROUND

JM101 is a recombination- and *Eco*K restriction-proficient (RecA$^+$, Hsdr$^+$) host strain.

JM101 Genotype: *supE thi-1* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}*ZΔM15*]. (Genes listed signify mutant alleles. Genes on the F′ episome, however, are wild-type unless indicated otherwise.)

JM101 cells contain the *lacI*^{q}*ZΔM15* gene on the F′ episome, allowing blue-white screening for recombinant plasmids.

TRANSFORMATION PROTOCOL

1. Pre-chill two 14-ml BD Falcon polypropylene round-bottom tubes on ice. (One tube is for the experimental transformation and one tube is for the pUC18 control.) Preheat SOC medium[§] to 42°C.
2. Thaw the competent cells on ice. When thawed, gently mix and aliquot 100 µl of cells into each of the two pre-chilled tubes.
3. Add 1.7 µl of β-mercaptoethanol provided with this kit to each aliquot of cells.
4. Swirl the contents of the tubes gently. Incubate the cells on ice for 10 minutes, swirling gently every 2 minutes.
5. Add 0.1–50 ng of the experimental DNA (see *Quantity and Volume of DNA*, reverse page, for guidelines) to one aliquot of cells and add 1 µl of the pUC18 control DNA to the other aliquot. Swirl the tubes gently.
6. Incubate the tubes on ice for 30 minutes.
7. Heat-pulse the tubes in a 42°C water bath for 45 seconds. The duration of the heat pulse is critical for maximum efficiency.
8. Incubate the tubes on ice for 2 minutes.
9. Add 0.9 ml of preheated SOC medium and incubate the tubes at 37°C for 1 hour with shaking at 225–250 rpm.
10. Plate ≤200 µl of the transformation mixture on LB agar plates containing the appropriate antibiotic (and containing IPTG and X-gal if color screening is desired).[§] For the pUC18 control transformation, plate 5 µl of the transformation on LB–ampicillin agar plates.[§]

 Notes *Cells may be concentrated by centrifuging at 1000 rpm for 10 minutes. Resuspend the pellet in 200 µl of SOC medium.*

 If plating <100 µl of cells, pipet the cells into a 200 µl pool of SOC medium and then spread the mixture with a sterile spreader. If plating ≥100 µl, the cells can be spread on the plates directly. Tilt and tap the spreader to remove the last drop of cells.

 Some β-galactosidase fusion proteins are toxic to the host bacteria. If an insert is suspected to be toxic, plate the cells on media without X-gal and IPTG. Color screening will be eliminated, but lower levels of the potentially toxic protein will be expressed.
11. Incubate the plates at 37°C overnight (at least 17 hours for blue-white color screening). Colonies containing plasmids with inserts will remain white, while colonies containing plasmids without inserts will be blue. The blue color can be enhanced by incubating the plates for two hours at 4°C following the overnight incubation at 37°C.
12. For the pUC18 control, expect 50 colonies (≥1 × 10^8 cfu/µg pUC18 DNA). For the experimental DNA, the number of colonies will vary according to the size and form of the transforming DNA, with larger and non-supercoiled DNA producing fewer colonies.

[§]See *Preparation of Media and Reagents*.

Transformation Guidelines and Troubleshooting

Storage Conditions: Competent and supercompetent cells are very sensitive to even small variations in temperature and must be stored at the bottom of a –80°C freezer. Transferring tubes from one freezer to another may result in a loss of efficiency.

Use of 14-ml BD Falcon polypropylene round-bottom tubes: It is important that 14-ml BD Falcon polypropylene round-bottom tubes (BD Biosciences Catalog #352059) are used for the transformation protocol, since other tubes may be degraded by β-mercaptoethanol. In addition, the duration of the heat-pulse is critical and has been optimized for these tubes.

Aliquoting Cells: Keep the cells on ice at all times during aliquoting. It is essential that the polypropylene tubes are placed on ice before the cells are thawed and that the cells are aliquoted directly into pre-chilled tubes. It is also important to use the volume of cells indicated in step 2 of the *Transformation Protocol*. Decreasing the volume will result in lower efficiencies.

Use of β-Mercaptoethanol (β-ME): β-ME has been shown to increase transformation efficiency. The β-ME provided is diluted and ready to use. A fresh 1:10 dilution (from a 14.2 M stock) may be used; however, Stratagene cannot guarantee results with β-ME from other sources.

Quantity and Volume of DNA: The greatest efficiency is obtained from the transformation of 1 µl of 0.1 ng/µl supercoiled pUC18 DNA per reaction. A greater number of colonies may be obtained by transforming up to 50 ng DNA, although the resulting efficiency (cfu/µg DNA) may be lower. The volume of the DNA solution added to the reaction may be increased to up to 10% of the reaction volume, but the transformation efficiency may be reduced.

Heat Pulse Duration: Optimal transformation efficiency is observed when cells are heat-pulsed at 42°C for 45–50 seconds. Efficiency decreases sharply when cells are heat-pulsed for <45 seconds or for >60 seconds.

Blue-White Color Screening: Blue-white color screening for recombinant plasmids is available when transforming host strains that contain the *lacI*^{q}*ZΔM15* gene on the F′ episome with a plasmid that provides α-complementation (e.g. Stratagene's pBluescript® II vector). When performing blue-white color screening, incubate the LB agar plates containing IPTG and X-gal at 37°C for at least 17 hours to allow color development. The blue color can be enhanced by subsequent incubation of the plates for two hours at 4°C.

Preparation of Media and Reagents

SOB Medium (per Liter)
20.0 g of tryptone
5.0 g of yeast extract
0.5 g of NaCl
Add deionized H_2O to a final volume of 1 liter
Autoclave
Add 10 ml of filter-sterilized 1 M $MgCl_2$ and 10 ml of filter-sterilized 1 M $MgSO_4$ prior to use

SOC Medium (per 100 ml)
Note This medium should be prepared immediately before use.
2 ml of filter-sterilized 20% (w/v) glucose or 1 ml of filter-sterilized 2 M glucose
SOB medium (autoclaved) to a final volume of 100 ml

LB Agar (per Liter)
10 g of NaCl
10 g of tryptone
5 g of yeast extract
20 g of agar
Add deionized H_2O to a final volume of 1 liter
Adjust pH to 7.0 with 5 N NaOH
Autoclave

LB–Ampicillin Agar (per Liter)
1 liter of LB agar, autoclaved
Cool to 55°C
Add 10 ml of 10 mg/ml filter-sterilized ampicillin
Pour into petri dishes (~25 ml/100-mm plate)

Preparation of Agar Plates for Blue-White Color Screening

To prepare plates for blue-white screening, prepare LB agar as indicated above. When adding the antibiotic, also add 5-bromo-4-chloro-3-inodlyl-β-D-galactopyranoside (X-gal) to a final concentration of 80 µg/ml [prepared in dimethylformamide (DMF)] and isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 20 mM (prepared in sterile dH_2O). Alternatively, 100 µl of 10 mM IPTG and 100 µl of 2% X-gal may be spread on solidified LB agar plates 30 minutes prior to plating the transformations. (For consistent color development across the plate, pipet the X-gal and the IPTG into a 100-µl pool of SOC medium and then spread the mixture across the plate. Do not mix the IPTG and the X-gal before pipetting them into the pool of SOC medium because these chemicals may precipitate.)

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Plasmid Stability in Immobilized and Free Recombinant *Escherichia coli* JM105(pKK223-200): Importance of Oxygen Diffusion, Growth Rate, and Plasmid Copy Number

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Stability of the plasmid pKK223-200 in *Escherichia coli* JM105 was studied for both free and immobilized cells during continuous culture. The relationship between plasmid copy number, xylanase activity, which was coded for by the plasmid, and growth rate and culture conditions involved complex interactions which determined the plasmid stability. Generally, the plasmid stability was enhanced in cultured immobilized cells compared with free-cell cultures. This stability was associated with modified plasmid copy number, depending on the media used. Hypotheses are presented concerning the different plasmid instability kinetics observed in free-cell cultures which involve the antagonistic effects of plasmid copy number and plasmid presence on the plasmid-bearing/plasmid-free cell growth rate ratio. Both diffusional limitation in carrageenan gel beads, which is described in Theoretical Analysis of Immobilized-Cell Growth, and compartmentalized growth of immobilized cells are proposed to explain plasmid stability in immobilized cells.

Plasmid stability and high biomass productivity are important factors for the development and commercial exploitation of genetically engineered cells, especially in food and chemical industries, in which the engineering challenge and production efficiencies are crucial (9, 39). Future strategies for synthesis of protein products by using recombinant host cells should involve the following factors (2): molecular control systems, single-cell kinetics, microbial production dynamics, reactor productivity, and downstream processing.

The following three levels of plasmid instability could correspond to the first three factors: structural instability (16), segregational instability (20), and competition instability (32). Enzyme engineering, through immobilization systems, already offers certain advantages corresponding to the latter two factors: higher productivities due to high cell concentration obtained in the support (8, 10), lower investment cost (18), easier downstream processing in terms of separation of product from cells (4), and also some physiological changes in cells (19, 29, 38).

In a previous work (7), we combined the methods of both genetic and enzyme engineering to overcome the difficulties in growing recombinant host cells. The immobilization of growing recombinant *Escherichia coli* cells led to an increased plasmid stability which could be explained only partially by the compartmentalization of the cell growth in the support.

The aim of the present study was to determine the role of cell immobilization on parameters involved in plasmid instability, such as the growth rate and the plasmid copy number. The target protein synthesized by the plasmid was an endo-β-D-xylanase (EC 3.2.1.8). Xylan is a β-1,4-linked heteropolymer of xylose, often with 4-*O*-methylglucuronate and arabinose side chains, and is located in cell walls of higher plants, especially hardwoods and grasses (37). Xylan hydrolysis could be of commercial significance, since some industrial processes, for example in the pulp and paper industry, release large quantities of xylan in their effluents which could be used as feedstock for bacterial protein production. Furthermore, xylanase could also be used to remove hemicellulose from hardwoods for possible application in dissolving pulp (24).

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MATERIALS AND METHODS

Bacterial strain and plasmid. A gene coding for endo-β-D-xylanase synthesis in *Bacillus subtilis* PAP115 was originally cloned in *E. coli* WA802 via the plasmid pBR325 (3). An *Eco*RI-bordered fragment of chromosomal *B. subtilis* DNA was isolated and sequenced (23). Data showed that this fragment, harboring the xylanase activity, contained a signal sequence which allowed, in at least two different *E. coli* strains, the secretion of a functional endo-β-D-xylanase activity in the culture supernatant (25). This fragment of DNA was then inserted in the *Eco*RI site of the expression vector pKK223-3 (Pharmacia P-L Biochemicals, Inc., Milwaukee, Wis.) to obtain the plasmid pKK223-200. This vector contains both the *tac* promoter, which is activated by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to the medium, and the ampicillin resistance gene as genetic markers. The recombinant plasmid was introduced by transformation into *E. coli* JM105 (Pharmacia P-L Biochemicals, Inc.), a thiamine proline-less mutant. The new clone, named *E. coli* JM105(pKK223-200), produced larger amounts of extracellular xylanase than did the original xylanase-producing *E. coli* strain and was used throughout this work.

Immobilization procedure. Cells were immobilized as previously described (34) by using 2% carrageenan (E 407; Ceca, Carentan, France). The mixture, 1 ml of inoculum (approximately 10^9 cells ml^{-1}) and 9 ml of gel solution, was pumped and added dropwise to 0.3 M KCl solution. The

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initial cell concentration in gel beads was then approximately equal to 10^8 cells ml^{-1}. The spherical beads formed had a mean diameter of approximately 3 mm. When necessary, the gel beads could be dissolved in 0.1 M sodium citrate at 40°C.

Media and culture conditions. The LB medium contained, per liter of deionized water, 10 g of Bacto-Peptone, 10 g of NaCl, and 5 g of yeast extract (all from Difco Laboratories, Detroit, Mich.). The minimal broth contained, per liter of deionized water, 10.6 g of Minimal Broth Davis (Bioclass Laboratories), 1 g of glucose, and 10 mg of thiamine. Both media were supplemented with 0.1 M KCl to ensure better mechanical stability of the gel beads. When necessary, ampicillin and IPTG were added to the media at final concentrations of 30 $\mu g\ ml^{-1}$ and 0.2 mM, respectively, in order to ensure that all cells were plasmid bearing and to derepress xylanase synthesis (P^+ and P^- cells are plasmid-bearing and plasmid-free cells, respectively). Batch cultures were carried out in 500-ml baffled Erlenmeyer flasks, each containing 100 ml of medium. The cultures were agitated at 250 rpm and 37°C (Orbit Environ-Shaker; Lab-Line, Melrose Park, Ill.) for various periods of time. Continuous cultures were conducted by using a MultiGen model F-1000 fermentor (New Brunswick Scientific Co., Inc., New Brunswick, N.J.) with a working volume ranging from 150 to 260 ml (26). The medium was pumped (Masterflex Digi-Staltic pump; Cole Parmer, Chicago, Ill.) into the fermentor through an inlet at the top of the vessel, and the spent medium containing cells was removed to an overflow jar with the same pump by using a second head with a larger tubing and thus a higher potential flow rate. Thus, the working volume of the fermentor was fixed by the height of the outlet tubing inside the fermentor. The release of gel beads from the fermentor was prevented by a grid surrounding the outlet tubing. The temperature of the chemostat was maintained at 37°C by means of a built-in temperature controller. Aeration was 5 air volumes/liquid volume per min, and the inlet air was humidified by three humidifying columns.

Cell counting and percentage of P^+ cells. Xylanase activity on plates was detected by adding 1% of the soluble fraction of xylan (Sigma Chemical Co., St. Louis, Mo.) and 1.5% agar to the LB medium. Recombinant xylanase-producing clones were identified by the formation of a clear zone around the colony after enzymic digestion of the opaque xylan suspension in the agar. Cultures were diluted in sterile saline and plated on the xylan medium (five plates per dilution). The percentage of P^+ cells was determined by using the ratio colonies showing a clear zone/total number of colonies. Preliminary results showed that ampicillin resistance was significantly correlated with extracellular xylanase synthesis.

Plasmid copy number. After DNA extraction and agarose gel electrophoresis (27), the gels were stained with a 1 mg ml^{-1} ethidium bromide solution for 30 min, and measurements of each band density from negatives of gel photographs were made with a scanning densitometer (LKB 2202 Ultroscan laser densitometer; LKB, Bromma, Sweden) coupled to a microcomputer for peak integration. The molecular masses of the plasmid and the chromosome were 5.86×10^6 daltons and approximately 2.6×10^9 daltons, respectively. The plasmid copy number (n_p) was calculated as follows: $n_p = (M_c \cdot C_p)/M_p \cdot C_c \cdot$ percentage of P^+ cells), where M_p and M_c were the molecular masses of the plasmid and the chromosome, respectively, and C_p and C_c were the amounts of plasmid and chromosome DNA, respectively.

Determination of xylanase in supernatant. Xylanase activity was measured as previously described (23) and was expressed in micromoles $minute^{-1}$ (international units $milliliter^{-1}$). Reducing sugar concentrations were determined by the dinitrosalicylic acid method (21).

Diffusion and partition of oxygen and glucose in the carrageenan gel beads. The diffusion coefficients of oxygen and glucose were determined by measuring the kinetics of the absorption of glucose into the gel beads and the desorption of oxygen into the solution (35). In the case of glucose, 50 ml of glucose-free gel beads was mixed with 50 ml of glucose solution of known concentration (approximately 1 g $liter^{-1}$) in an Erlenmeyer flask. The decrease in glucose concentration in the solution, which was due to the diffusion of glucose into the gel beads, was measured repeatedly by the dinitrosalicylic acid method (21). In the case of oxygen, the beads were oxygen saturated in oxygen-saturated water. The initial oxygen concentration in gel beads was 6.3×10^{-3} g $liter^{-1}$. A 45-ml amount of oxygen-saturated gel beads was mixed with 40 ml of oxygen-free water. The increase in oxygen concentration in the solution was determined on a continuous basis by an oxygen meter (model 54A; Yellow Springs Instrument Co., Yellow Springs, Ohio).

The partition coefficients were determined by measuring the ratio of glucose and oxygen concentrations in the beads to the respective concentrations in the solution at equilibrium.

The experiments were performed at a temperature of 37°C and at an agitation of 250 rpm (Lab-Line Orbit Environ-Shaker).

THEORETICAL ANALYSIS OF IMMOBILIZED-CELL GROWTH

Determination of oxygen and glucose diffusion and partition coefficients in gel beads. The diffusion coefficients of glucose and oxygen in carrageenan beads were determined by using the equations developed by J. Crank (5).

In the case of glucose, the gel beads were initially glucose free. The glucose concentration in the solution (C_L) was initially C_{L0} and is defined by the following expression:

$$C_L = \frac{\alpha C_{L0}}{1+\alpha}\left\{1 + \sum_{n=1}^{\infty} \frac{[6(1+\alpha)]e^{-Dq_n^2 t/a^2}}{9 + 9\alpha + q_n^2\alpha^2}\right\}$$

where a is the radius of beads, D is the diffusion coefficient of glucose, and q_n is the nonzero positive root of the following equation: $\tan q_n = 3q_n/(3 + \alpha q_n^2)$, where α is the ratio of solution to bead volume corrected by the partition coefficient K:

$$\alpha = \frac{1}{K}\frac{V_L}{V_B} = \frac{3V_L}{m_B\, 4\pi\, a^3\, K}$$

where V_L is the volume of the solution without the beads, V_B is the total volume of the beads, and m_B is the number of beads. It can be shown also that K is defined by $K = C_{Be}/C_{Le}$, where C_{Be} and C_{Le} are the concentrations, at equilibrium, of solute in the beads and in the solution, respectively.

In the case of oxygen, the solution was initially oxygen free, and the oxygen concentration in the beads was initially C_{B0}. The oxygen concentration in the solution is defined by the following expression:

$$C_L = \frac{C_{B0}}{1+\alpha}\left\{1 - \sum_{n=1}^{\infty} \frac{[6\alpha(1+\alpha)]e^{-Dq_n^2 t/a^2}}{9 + 9\alpha + q_n^2\alpha^2}\right\}$$

PAGE 28/41 * RCVD AT 10/26/2007 2:41:20 PM [Eastern Daylight Time] * SVR:USPTO-EFXRF-3/20 * DNIS:2738300 * CSID: * DURATION (mm-ss):15-52

TABLE 1. Kinetic characteristics of P^+ and P^- cells in minimal and LB media

Plasmid status of cell	Kinetic characteristics[a] in:				
	Minimal medium				LB medium (μ_{max} [h^{-1}])
	μ_{max} (h^{-1})	Q_{max} (h^{-1})	$Y_{X/glc}$	$K_{S_{glc}}$ (mg liter^{-1})	
P^+	0.55 ± 0.02	0.57 ± 0.02	0.96 ± 0.07	9 ± 3	0.85 ± 0.02
P^-	0.63 ± 0.02	0.39 ± 0.02	1.61 ± 0.07	52 ± 15	1.02 ± 0.02

[a] μ_{max} is the maximum specific growth rate. Q_{max} is the maximum specific glucose consumption rate. $Y_{X/glc}$ is the yield factor of biomass growth to glucose consumed (μ_{max}/Q_{max}), and $K_{S_{glc}}$ is the saturation constant of glucose (glucose concentration at $\mu = \mu_{max}/2$). Data are means ± standard deviations.

The experimental results were fitted by using the least-square method.

Kinetics of cell growth and substrate consumption. The growth rate was determined by using the double-substrate Monod equation kinetics:

$$\mu = \mu_{max}\left(\frac{[O_2]}{[O_2] + K_{S_{O_2}}}\right)\left(\frac{[Glc]}{[Glc] + K_{S_{Glc}}}\right)$$

where μ_{max} is the maximum specific growth rate, $[O_2]$ and [Glc] are the concentrations of oxygen and glucose, respectively, and $K_{S_{O_2}}$ and $K_{S_{Glc}}$ are the saturation constants of oxygen and glucose, respectively.

The rates of substrate consumption (Q) were calculated as follows: $Q_{O_2} = \mu/Y_{X/O_2}$ and $Q_{Glc} = \mu/Y_{X/Glc}$, where Y_{X/O_2} and $Y_{X/Glc}$ are the stoichiometric constants of biomass produced to oxygen and glucose consumed, respectively.

During a period of time Δt, the biomass concentration (X) increases as follows: $X_{(t+\Delta t)} = X_t \exp^{\mu\Delta t}$. The quantity of substrate utilized for biomass growth was calculated as follows:

$dS/dt = QX$, which is solved by $\Delta S = QX\Delta t$, and thus

$$\Delta[O_2] = Q_{O_2}X\Delta t \text{ and } \Delta[Glc] = Q_{Glc}X\Delta t$$

Kinetics of diffusion and consumption of substrates. Glucose and oxygen diffuse into the beads and are both consumed for biomass growth according to the kinetics described above. Here, we used a numerical method to solve the diffusion equation (finite-difference approximation) (5). The oxygen and glucose concentrations in the beads were initially equal to zero, and the surface concentrations of both substrates were constants. The following nondimensional variables were introduced: $R = r/a$ and $T = D(t/a^2)$, and thus $\Delta R = \Delta r/a$ and $\Delta T = D(\Delta t/a^2)$.

On using these variables, the equation for radial diffusion in a sphere of radius a becomes $\partial C/\partial T = \partial^2 C/\partial R^2 + 2\partial C/R\partial R$.

By using the finite difference approximation, we obtained the following diffusion kinetics:

$$C_{(t+1,\,m)} = C_{(t,\,m)} + \left\{\frac{D\Delta t}{m(\Delta R)^2}\left[(m+1)C_{(t,\,m-1)} - 2mC_{(t,\,m)} + (m+1)C_{(t,\,m-1)}\right]\right\}$$

and, for $m = 0$ (at the centers of beads),

$$C_{(t+1,\,m)} = C_{(t,\,m)} + \frac{6D\Delta t}{(\Delta R)^2}\left(C_{(t,\,m+1)} - C_{(t,\,m)}\right)$$

where m is the number of intervals which divide the radius of the beads and $C_{(t,\,m-1)}$, $C_{(t,\,m)}$, and $C_{(t,\,m+1)}$ denote the substrate concentrations at the points $(m-1)\Delta r$, $m\Delta r$, and $(m+1)\Delta r$, respectively, at time t. $C_{(t+1,\,m)}$ is the substrate concentration at the point $m\Delta r$ at time $t + \Delta t$.

Each concentration was then modified at each time and each radial position on the basis of the previously described kinetics of cell growth and substrate consumption. The value of $D\Delta t/(\Delta r)^2$ chosen was ½ and the number of intervals dividing the beads was $m = 80$. Calculations were made by using a microcomputer.

RESULTS

Kinetic characteristics of free P^+ and P^- cells. Batch fermentations were used to determine the kinetic characteristics of free P^+ and P^- cells. The results are shown in Table 1. For the P^+ cell cultures, ampicillin was added to the media in order to maintain 100% P^+ cells. In the minimal media cultures the optical density of the cell suspension at 600 nm and the glucose concentration were monitored. For P^+ and P^- cells, the maximum specific growth rates (μ_{max}) and the maximum glucose consumption rate (Q_{max}) were calculated by measuring the slopes of ln optical density at 600 nm versus time plots and the substrate consumption rate versus biomass concentration ($dS/dt = -Q_{max} \cdot X$), respectively; the glucose saturation constants ($K_{S_{Glc}}$) were determined by using Lineweaver-Burk plots. The yield $Y_{X/Glc}$ is the ratio μ_{max}/Q_{max} and represented energy needed for both cell growth and cell maintenance. The μ_{max} P^+/μ_{max} P^- ratios in LB and minimal media were found to be 0.83 ± 0.04 and 0.87 ± 0.06, respectively.

Relationship between plasmid copy number, extracellular xylanase activity, and growth rate. Continuous free-cell cultures in LB and minimal media were conducted at different dilution rates. IPTG and ampicillin were added to both media in order to obtain xylanase production and to maintain 100% P^+ cells, respectively. The cultures were maintained at desired dilution rates during a 24-h period in order for them to reach their steady state, and then plasmid copy number and extracellular xylanase activity were determined. In minimal media (Fig. 1a), the plasmid copy number increased when the dilution rate, which represents the growth rate of the cells, decreased. The effect was less significant in LB medium. Furthermore, at the same dilution rates (between 0.3 and 0.5 h^{-1}), the plasmid copy number was dependent on the medium used. Despite the sharp increase in plasmid copy number in minimal media (Fig. 1b), the extracellular xylanase activity remained approximately constant at 0.3 IU ml^{-1}; contrast, the relatively low increase in plasmid copy number in LB media was associated with an important

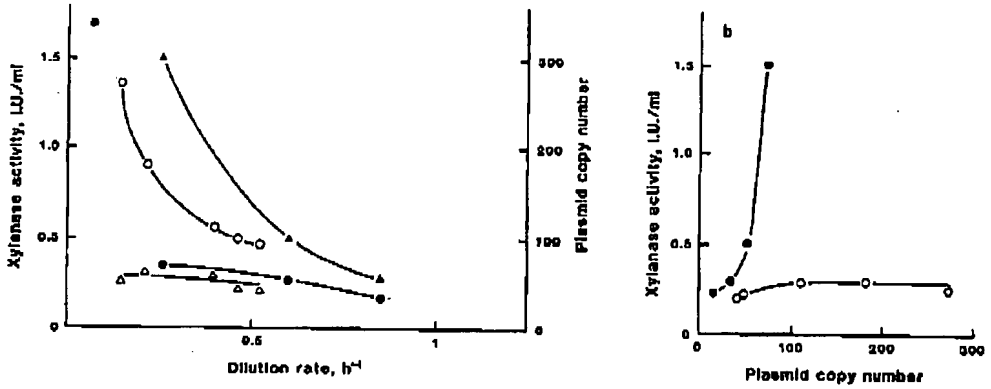


FIG. 1. Relationship between plasmid copy number, extracellular xylanase activity, and dilution rate in free-cell continuous cultures with selection pressure. (a) Xylanase activity in LB (▲) and minimal medium (△) and plasmid copy number in LB (●) and minimal medium (○). (b) Xylanase activity in LB (●) and minimal medium (○). Ampicillin and IPTG were added to both media in order to obtain 100% P^+ cells and xylanase synthesis. The continuous cultures were maintained at the desired dilution rates during a 24-h period in order to allow them to reach their steady state, and then plasmid copy number and extracellular xylanase activity were determined.

increase of up to 1.5 *IU* ml^{-1} in extracellular xylanase activity.

Plasmid stability in free-cell continuous cultures. In the present study, the continuous cultures were run without ampicillin, and only LB medium was supplemented with IPTG. The number of generations was calculated from $D_{rate}\,t/\ln 2$ (17), where D_{rate} is the dilution rate and t is the time.

By using minimal media, two continuous cultures were conducted at dilution rates of 0.19 h^{-1} (35% of μ_{max} P^+ in minimal media) and 0.46 h^{-1} (84% of μ_{max} P^+ in minimal media). The plasmid stabilities and plasmid copy numbers are shown in Fig. 2a and b. The culture conducted at the higher dilution rate showed a higher plasmid stability compared with the one conducted at the lower dilution rate. However, the characteristics of these two curves were different: at the lower growth rate, during the first 40 generations, the percentage of P^+ cells and the plasmid copy number remained stable. Subsequently, the percentage of P^+ cells began to decrease, and at the same time the plasmid copy number increased to 200. Concurrently, in the higher-dilution-rate culture, the percentage of P^+ cells and the plasmid copy number started to decrease immediately.

A continuous culture was conducted by using LB medium at a dilution rate of 0.76 h^{-1} (90% of the μ_{max} P^+ in LB media). The plasmid copy number, the extracellular xylanase activity, and the percentage of P^+ cells for both cultures are shown in Fig. 3a and b. The percentage of P^+ cells in the free-cell culture started to decrease immediately and significantly faster than in the higher-dilution-rate free-cell culture in minimal media. The plasmid copy number curve showed the same shape as that of the xylanase activity, and both became undetectable quickly.

Plasmid stability in immobilized-cell continuous cultures.

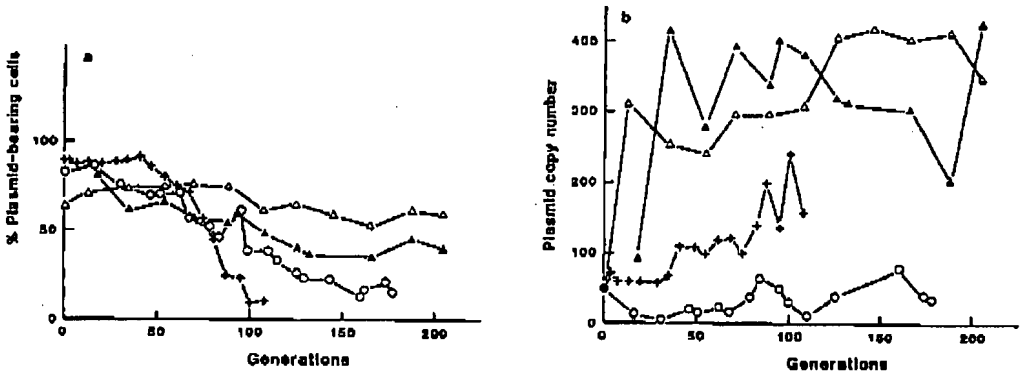


FIG. 2. Continuous cultures of free and immobilized cells in minimal medium without selection pressure. (a) Percentage of P^+ cells in cultures of immobilized cells (△), cells released from gel beads, (▲), and free-cell cultures at D_{rate} = 0.19 h^{-1} (+) and D_{rate} = 0.46 h^{-1} (○). (b) Plasmid copy number per P^+ cell in immobilized cells (△), cells released from gel beads (▲), and free-cell cultures at D_{rate} = 0.19 h^{-1} (+) and D_{rate} = 0.46 h^{-1} (○). No ampicillin was added to the medium, and the dilution rate of the immobilized-cell culture (D_{rate} = 1.4 h^{-1}) was made much higher than the μ_{max} of P^- and P^+ cells in order to wash out the cells released from the beads and thereby to avoid their division in the fermentor. The number of generations was calculated from ($D_{rate}\,t/\ln 2$ for the free-cell cultures and (μ_{max} P^- $t/\ln 2$ for the immobilized-cell culture (17).

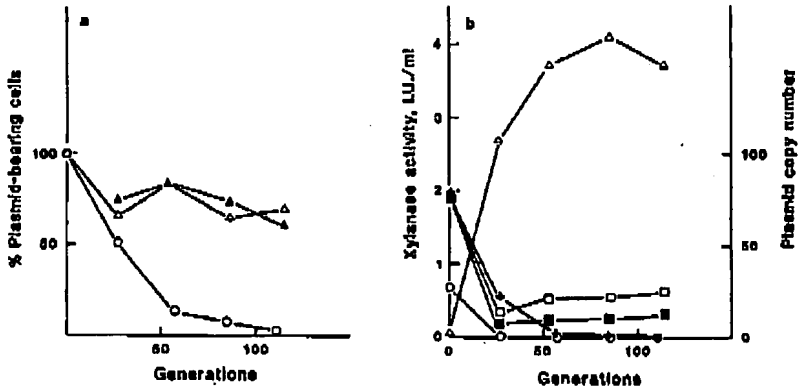


FIG. 3. Continuous cultures of free and immobilized cells cultured in LB medium without selection pressure. (a) Percentage of P^+ cells in immobilized cells (△), cells released from gel beads (▲), and a free-cell culture at $D_{rate} = 0.76\ h^{-1}$ (○). (b) Plasmid copy number per P^+ cell in immobilized cells (□), cells released from gel beads (■), and a free-cell culture at $D_{rate} = 0.76\ h^{-1}$ (•). Xylanase activity of immobilized cells (△) and a free-cell culture at $D_{rate} = 0.76\ h^{-1}$ (○). The LB media contained IPTG but no ampicillin. The dilution rate of the immobilized-cell culture was $D_{rate} = 1.9\ h^{-1}$, and the generation number was calculated as described in the legend to Fig. 2.

The dilution rates of the immobilized-cell cultures were made much higher than the maximum growth rate of both P^+ and P^- cells in order to wash out the cells released from the beads and thereby to avoid their being divided in the fermentor. The number of generations was calculated from the equation $\mu_{max}\ P^+\ t/\ln 2$.

In minimal media culture (Fig. 2a and b), the percentage of P^+ cells in the beads remained approximately constant (less than 10% P^+ cell loss during 200 generations). The percentage of P^+ cells released from gel beads showed the same curve as the immobilized cells but had a 10 to 20% lower value. The plasmid copy number of both P^+ cells in the beads and those released from the beads rose to the 400 level.

In the LB media culture (Fig. 3a and b), the percentage of P^+ cells began to decrease, to 75%, and remained constant until the end of the experiment. The plasmid copy number in both immobilized and released cells decreased during the first 25 generations and then remained constant at values of 20 and 10 copies, respectively. On the other hand, the extracellular xylanase activity measured in gel beads, after their dissolution, increased to 4 IU ml^{-1}.

Another immobilized-cell culture in LB medium showed a stable maintenance of the percentage of P^+ cells, approximately equal to 100% in both immobilized and released cells, until generation 500, at which point the experiment was stopped (data not shown).

Cell growth in carrageenan gel beads. The diffusion coefficients of oxygen and glucose at 37°C in 2% carrageenan gel with 0.1 M KCl were found to be equal to $1.58 \times 10^{-9}\ m^2\ s^{-1}$ and $9.81 \times 10^{-10}\ m^2\ s^{-1}$, respectively. The oxygen diffusion coefficient can be given as a percentage of its value in pure water at the same temperature. The oxygen diffusion coefficient in pure water at 25°C is $2.41 \times 10^{-9}\ m^2\ s^{-1}$; it was calculated at 37°C by using the equation $D = \text{const}\ \Theta/\eta$ (1), where Θ is the temperature in degrees Kelvin and η is the viscosity of water at the defined temperature. On the basis of this equation, the oxygen diffusion coefficient in water at 37°C is $3.23 \times 10^{-9}\ m^2\ s^{-1}$. Thus, the oxygen diffusion coefficient in 2% carrageenan gel is 49% of its value in pure water and so is lower than in calcium alginate or agar beads (1, 31) but higher than in barium alginate beads (14). The partition coefficients for oxygen and glucose were 0.7 and 1.0, respectively. This indicates that there was no partition for glucose and that the partition coefficient for oxygen was not favorable for the carrageenan gel.

The diffusion-consumption model was developed by using the values of parameters shown in Table 2. They correspond to a continuous culture of immobilized P^+ cells in minimal media. The plasmid instability was not taken into account in the model. The oxygen and glucose concentrations in the bulk solution were constant. As described in a previous experiment (7), the biomass growth was limited to the outer layer of beads. The simulation of biomass growth showed that 74% of the entire biomass contained in beads was found in a 150-μm outer layer of beads which represents 25% of the total bead volume. This limited growth was due to the lack of oxygen in the centers of beads while they were continuously saturated with glucose. This oxygen limitation in immobi-

TABLE 2. Parameter values[a] for simulation of biomass growth in gel beads

Parameter	Unit	Value
μ_{max}	(h^{-1})	0.55
$K_{S_{O_2}}$	(g $liter^{-1}$)	7.5×10^{-3}
$K_{S_{Glc}}$	(g $liter^{-1}$)	9×10^{-3}
Y_{X/O_2}		1.33
$Y_{X/Glc}$		0.96
D_{O_2}	($m^2\ s^{-1}$)	1.58×10^{-9}
D_{Glc}	($m^2\ s^{-1}$)	9.81×10^{-10}
K_{O_2}		0.7
K_{Glc}		1
a	(mm)	1.56
[Glc]	(g $liter^{-1}$)	1
$[O_2]$	(mg $liter^{-1}$)	4.25

[a] K_{O_2} and K_{Glc} are the partition coefficients of oxygen and glucose, respectively; a is the radius of the beads; [Glc] and $[O_2]$ are the constant concentrations of glucose and oxygen in the bulk solution (multiplied by K_{Glc} and K_{O_2}, respectively); and D_{O_2} and D_{Glc} are the diffusion coefficients of oxygen and glucose, respectively, in the carrageenan gel. The other parameters are defined in Table 1, footnote *a*. The values of Y_{X/O_2} and $K_{S_{O_2}}$ are assumed from reference 36.

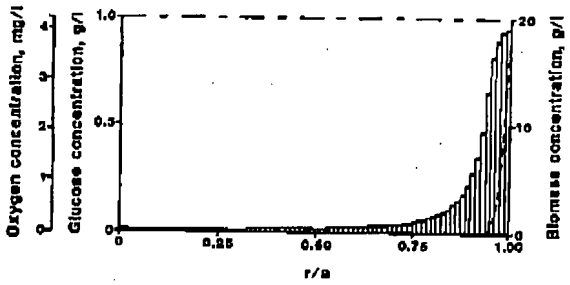


FIG. 4. Theoretical profiles of immobilized-cell growth. Shown are glucose concentration (— — —), oxygen concentration (– – – –), and a histogram of biomass concentration after 13 h of simulated growth. The abscissa represents the distance from the surface of the beads. The left side of the figure represents the center of the bead, and the right side represents the surface. The histogram of biomass concentration is divided into 80 intervals, each one corresponding to 19.5 μm. The values of parameters used in the model are shown in Table 2.

lized cells (11, 28, 36) after 13 h of simulated growth is shown in Fig. 4. At this time, cell-containing cavities in the outer layers of beads explode, due to the cell growth, and cells are released from beads. The growth rate of immobilized cells depended on their location in the beads. Consequently, their plasmid copy number could change on the basis of their growth rate.

DISCUSSION

From the single-cell kinetics point of view, plasmid-free cells have a growth rate advantage over plasmid-bearing cells, as observed in numerous cases (15). The ratio μ_{max} P^+/μ_{max} P^- is approximately 0.85 and does not significantly change in either LB or minimal medium. Thus, the competitive advantage of P^- cells is the same at different growth rates. This difference in growth rate could be due to the additional energy needed for replication and transcription of the plasmid (22, 30, 33) on the basis of the higher yield factor $Y_{X/Glc}$ determined for P^- cells rather than for P^+ cells. More surprising is the result concerning the glucose saturation constant of P^+ cells, which was found to be approximately six times lower than that of P^- cells and which thus allowed a growth advantage for P^- cells at low glucose concentrations (6).

The study of the relationships between the dilution rate, the plasmid copy number, and the extracellular xylanase activity was done with ampicillin selection pressure to avoid the problem of plasmid instability. As a general observation, the dilution rate of free-cell continuous cultures influences the plasmid copy number of cells. However, the characteristics of curves obtained in LB media (low increase) and in minimal media (exponentiallike increase) are different. Furthermore, for the same range of dilution rates, the plasmid copy numbers are different depending on the media used. These results show that the plasmid copy number does not correlate exclusively with the dilution rate of the culture but could also involve, for example, the nature of the growth-limiting factor, which is glucose for the minimal medium and which has not been determined in the more complex LB medium. On the other hand, extracellular xylanase production not only is associated with the plasmid copy number but also depends on the media used. Despite the large increase in plasmid copy number in minimal medium, the extracellular xylanase activity remained almost constant. In the LB culture, a relatively low increase in plasmid copy number invoked a large increase in xylanase activity. Xylanase gene transcription efficiency or, in a more general term, a lack of available energy for P^+ cells could explain this result. However, it can be observed that this lack of available energy does not result in a lower rate of plasmid replication. These results show that, for the enzyme production, both plasmid copy number and culture conditions are important factors to take into account (32).

In all free-cell continuous cultures without antibiotic selective pressure, the plasmids are not stably maintained in terms of percentage of P^+ cells. The determined percentage of P^+ cells involves two different factors: the probability that a P^- cell appears at cell division and, once this P^- cell appears, the competition between P^+ and P^- cells. The plasmid copy number and the P^+/P^- cell growth rate ratio could be the two parameters which correspond to these factors and could have antagonistic effects on plasmid stability. First, if the plasmid copy number increases the probability that a P^- cell will appear at cell division decreases, but the difference in growth rate of P^+ and P^- cells increases, allowing the P^- cells to rapidly overgrow the culture. Second, if the plasmid copy number is low the overgrowth of P^- cells would be slower, but the probability that a P^- cell will appear at cell division could increase. These two cases could correspond to the two different shapes of plasmid instability curves described in the Results section of this paper: (i) an initial plasmid stability followed by a fast decrease in stability or (ii) an initial decrease in plasmid stability, respectively. Thus, on the basis of these results, the plasmid copy number increases in low-dilution-rate cultures without antibiotics but this increase is not sufficient to maintain a constant percentage of P^+ cells due to a possible increased difference between P^+ and P^- cell growth rates.

Immobilized-cell continuous cultures in both LB and minimal media showed a higher plasmid stability than did free-cell cultures. In the case of the immobilized-cell culture conducted in the minimal media, this stability is associated with a very high plasmid copy number. In contrast to the free-cell culture, the high copy number in the immobilized-cell culture does not produce a large decrease in the percentage of P^+ cells. The immobilization involves a compartmentalized type of cell growth which limits the competition between P^+ and P^- cells (7) and therefore provides a higher plasmid stability. In fact, the determined plasmid copy number of immobilized cells is an average copy number because of the gradient of growth rates and consequently the plasmid copy number of cells in the beads. It can be postulated that the cells in the central part of beads have more than 400 plasmid copies. Furthermore, because of the "movement" of cavities to the surfaces of beads, which is due to the explosion of the outermost cavities and the subsequent release of cells into the bulk solution, P^+ cells which began to grow at a low growth rate, due to oxygen limitation, and which thus accumulated high plasmid copy number could then grow at higher rates and at the same time maintain the percentage of P^+ cells at a relatively stable level for several generations. Thus, in contrast to free cells, the immobilized cells show a history until the explosion of the cavities. In the immobilized-cell culture grown in LB medium, the xylanase activity obtained in gel beads reflects the enhanced plasmid maintenance. This extracellular xylanase activity could be not only xylanase production by P^+ cells

but also the activity of xylanase proteins which were retained in the beads. However, the molecular weight of this xylanase is 22,000 (3) and is too low for the protein to have been trapped inside the beads, unless the gel structure changes due to compression (7) and/or chemical reactions of metabolites excreted by immobilized cells. The intracellular xylanase activity was not studied specifically, but punctual experiments in both batch and continuous cultures showed an extracellular/intracellular xylanase activity ratio approximately equal to one. As was shown by another continuous culture of immobilized cells in LB medium during 500 generations, the enhanced plasmid stability can be maintained over a long fermentation time.

Both diffusion limitation, which produces a gradient of cell growth rates (and also of plasmid copy number), and compartmentalization of the cell growth could be involved in the higher plasmid stability obtained in immobilized cells. Furthermore, despite the oxygen diffusion limitation, the biomass and enzyme productivities of immobilized-cell continuous cultures are higher than those of free-cell cultures because of the high cell concentration in the support. Thus, gel beads can be a dynamic reserve of highly concentrated P^+ cells having a high plasmid copy number and being partially saved from competition with P^- cells, therefore providing for an enhanced plasmid stability. As was previously shown for enzyme reactions (12, 13), when phenomena such as reaction, diffusion, and/or partition are involved in an immobilized-cell system as opposed to a homogeneous one, such effects as spatial or temporal divisions and periodic and/or qualitatively different behaviors can be observed.

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Bacteriophage T4 DNA Replication Protein 41

CLONING OF THE GENE AND PURIFICATION OF THE EXPRESSED PROTEIN*

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The bacteriophage T4 primase, composed of the T4 proteins 41 and 61, synthesizes pentaribonucleotides used to prime DNA synthesis on single-stranded DNA *in vitro*. 41 protein is also a DNA helicase that opens DNA in the same direction as the growing replication fork. Previously, Mattson *et al.* (Mattson, T., Van Houwe, G., Bolle, A., Selzer, G., and Epstein, R. (1977) *Mol. Gen. Genet.* 154, 319–326) located part of gene 41 on a 3400-base pair *Eco*RI fragment of T4 DNA (map units 24.3 to 21.15). In this paper, we report the cloning of T4 DNA representing map units 24.3 to 20.06 in a multicopy plasmid vector. Extracts of cells containing this plasmid complement gene 41⁻ extracts in a DNA synthesis assay, indicating that this region contains all the information necessary for the expression of active 41 protein. We located gene 41 more precisely between T4 map units 22.01 to 20.06 since our cloning of this region downstream of the strong λ promoter P_L results in the production of active 41 protein at a level 100-fold greater than after T4 infection. We have purified 133 mg of homogeneous 41 protein from 27 g of these cells. Like the 41 protein from T4 infected cells, the purified 41 protein in conjunction with the T4 gene 61 priming protein catalyzes primer formation (assayed by RNA primer-dependent DNA synthesis with T4 polymerase, the genes 44/62 and 45 polymerase accessory proteins, and the gene 32 helix-destabilizing protein) and is a helicase whose activity is stimulated by T4 61 protein.

The bacteriophage T4 proteins encoded by the T4 gene 43 (DNA polymerase), gene 32 (helix-destabilizing protein), genes 44, 62, and 45 (polymerase accessory proteins), and genes 41 and 61 (priming proteins) together *in vitro* constitute an efficient system for DNA replication (1–3). Studies using these proteins, purified from the T4-infected cell, have helped to define the function of each of these T4 gene products in DNA synthesis. The T4 primase, composed of the 41 and 61 proteins, synthesizes pentaribonucleotides, of the sequence pppACN$_3$, which are used to prime DNA synthesis on single-stranded DNA (1, 2, 4–6). These primers are efficiently elongated by T4 DNA polymerase (in the presence of the polymerase accessory proteins 44/62 and 45) in a reaction analogous to DNA synthesis on the lagging strand. In addition, 41 protein is also a single-stranded DNA (ssDNA[1]) stimulated nucleotidase using rGTP, dGTP, rATP, or dATP (7–9) and a DNA unwinding enzyme (helicase) which is greatly stimulated by gene 61 protein (see Ref. 10 and this paper). The helicase activity requires a ssDNA extension from the 5′ end of the duplex which presumably acts as a binding site for the protein. The 41 helicase then removes the complementary strand beginning at its 3′ terminus (10). The finding that the 41/61 primase catalyzes both helicase and priming activities supports the proposal that the complex of 41 and 61 proteins, driven by NTP hydrolysis, participates in opening the growing replication fork as the primase moves 5′ to 3′ on the lagging strand template in search of appropriate priming sites (3, 10, 11). While such movement has not been demonstrated directly, the ability of 41 protein in the presence of 43, 44/62, 45 proteins and limiting amounts of 32 protein to stimulate the leading strand synthesis on a nicked duplex template (1, 10, 11) suggests that the T4 primase is involved in unwinding the helix ahead of the replication fork.

The coupling of helicase and primase activities has been implicated in prokaryotic replication systems besides T4 (for reviews, see Refs. 12 and 13). In bacteriophage T7, the primase, encoded by the T7 gene 4, is both a ssDNA-dependent nucleotidase and a helicase that synthesizes short oligoribonucleotides (predominantly pppACCC or pppACCA) to be used by the T7 DNA polymerase (14–16). In addition, the 5′ to 3′ movement of the gene 4 priming activity on ssDNA has been demonstrated directly (14), as well as inferred from its stimulation of leading strand synthesis using duplex templates (17). The helicase activity is inhibited by the nonhydrolyzable analog β,γ-methylene dTTP, suggesting that the movement of gene 4 protein is fueled by its nucleotidase activity (18). In *Escherichia coli*, the *dnaG* product has been assigned the priming activity based on its ability to synthesize an oligonucleotide primer on the ssDNA genomes of G4 and related phages coated with single-stranded DNA binding protein (SSB) (18, 19). In the absence of SSB protein, *dnaG* protein plus *dnaB* protein (a ssDNA-dependent nucleotidase) are sufficient *in vitro* for priming on many different ssDNA templates (20, 21). It has been proposed that, in this priming reaction, *dnaG* protein recognizes a structure formed by the binding of *dnaB* protein to ssDNA (20). Recently, McMacken *et al.* (22) have demonstrated that the *dnaB* protein is a helicase which opens duplex DNA in the same direction as the T4 41 protein and T7 gene 4 protein. Thus, a complex of the *dnaB* and *dnaG* proteins might be analogous to the T7 gene 4 or T4 41/61 primases. However, the priming complex (primosome) required to prime SSB-coated øøX174 DNA

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[1] The abbreviations used are: ssDNA, single-stranded DNA; bp, base pair; SDS, sodium dodecyl sulfate; T4 dC-DNA, cytosine-containing T4 DNA; SSB, single-stranded DNA binding protein.

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has been shown to involve several proteins besides those encoded by *dnaG* and *dnaB*, and the 5′ to 3′ movement of the primosome on this ssDNA apparently does not require the nucleotidase activity of *dnaB* protein (23). Thus, whether a primase composed of the *dnaB* and *dnaG* proteins behaves similarly to the T4 and T7 primases is not clear. In eukaryotic systems, priming activities which synthesize RNA primers of, on the average, 10 residues in length have been found to be tightly associated with DNA polymerase α from human, mouse, calf thymus, *Drosophila*, and *Xenopus*, and with DNA polymerase I from yeast (for a recent discussion see Ref. 24). Whether these primases, like the T4 and T7 primases, also exhibit helicase activity has not yet been reported.

We are interested in understanding the nature of the T4 primase and its interaction with the other T4 replication proteins. In order to obtain the large quantities of purified proteins that are needed for further biochemical studies, we have cloned gene 41 in a multicopy plasmid vector downstream of the strong λ promoter P_L. Cells carrying this recombinant plasmid produce approximately 100-fold more 41 protein than T4-infected cells. We have developed an efficient three-column purification procedure which yields 133 mg of homogeneous enzyme from 27 g of cells. In the accompanying paper (25), we describe our cloning of the gene for the other T4 priming protein, 61 protein.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Phage

Escherichia coli DH1, a Su^+, *rec*A strain which has a high efficiency for plasmid transformation, has been described (26). Other *E. coli* strains, containing the λ repressor cI, were used for the transformation of plasmids containing the λ promoter P_L: OR1265 (cI857) (27), the gift of K. Abremski and R. Hoess (Dupont) and N99cI$^+$, obtained from Pharmacia P-L Biochemicals. ER21 (Su^-) (28) and CR63 (Su^+) (29) were used for the marker rescue experiments. Unless otherwise indicated, cells were grown in L broth (10 g of Bacto-tryptone (Difco), 5 g of Bacto-yeast extract (Difco), and 5 g of NaCl/liter). T4D amN81 (gene 41) was obtained from W. Wood (University of Colorado), and T4D am4335 (gene 43) was obtained from J. Drake (National Institute of Environmental Health Sciences). T4 JW819, from J. Wiberg (University of Rochester School of Medicine and Dentistry), is T4D amE51X5 (gene 56), amN55X5 (gene 42), nd28 (gene denA), D2a2X5 (gene denB), and alc10 and was constructed as described for JW800 (30) except that the denB point mutant D2a2X5 was used. T4 JW819 was used with *E. coli* B834 *galU56* (31) for obtaining the cytosine-containing T4 DNA (T4 dC-DNA) used for the cloning experiments. (Wild type T4 DNA contains glycosylated, hydroxymethylcytosine residues which made it refractory to most restriction endonucleases.) Cytosine-containing T4 phage JW819 was grown as described (30) and the phage was purified by differential centrifugation followed by centrifugation in CsCl (32).

Construction of Plasmids

Cleared lysates containing plasmid DNA were prepared essentially as described (33) and the plasmid DNA was isolated after extraction of the lysate with phenol and chloroform and precipitation of the DNA in ethanol. T4 dC-DNA was released from purified phage essentially as described (34). The T4 DNA was precipitated in ethanol and spooled on a glass rod. After drying, both plasmid and phage DNAs were dissolved in TE buffer (10 mM Tris-HCl, pH 7.9, and 1 mM EDTA). DNA restriction fragments, obtained after electrophoresis of products on 0.8 or 1.5% agarose gels were isolated from the gel by electroelution (35). The NaCl concentration of the eluate was made 0.2 M and the eluate was applied to a RPC-5 analog column (0.2 ml) (Bethesda Research Laboratories) and eluted with a solution of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 2 M NaCl. The DNA was precipitated in ethanol, washed with 70 or 100% ethanol, dried, and redissolved in TE buffer.

Plasmids were constructed as follows. The vector DNA was incubated with the appropriate restriction enzyme, treated at 37 °C for 1 h with calf intestine alkaline phosphatase (0.02 to 0.3 unit/μg of DNA in a mixture containing 20 mM Tris-HCl, pH 7.9, and 10 mM $MgCl_2$), and gel-purified as described above. For ligations, 40 to 50 ng of vector DNA plus a 4- to 5-fold molar excess of the purified DNA fragment to be cloned were ligated at 0 °C overnight in a reaction mixture (20 to 30 μl) containing 30 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM ATP, 100 μg/ml bovine serum albumin, and 1 Weiss unit of T4 DNA ligase (Pharmacia P-L Biochemicals). The ligation mixture was extracted with an equal volume of phenol, and the DNA was precipitated with ethanol and redissolved in TE buffer. An aliquot of the ligated DNA or of supercoiled plasmid DNA (5 to 20 ng) was used to transform cells by either the procedure of Degert and Erlich (36) for DH1 or OR1265 (at 30 °C) or by the procedure of Hanahan (26) for N99cI$^+$. (Transformation efficiencies for DH1 and N99cI$^+$ cells ranged from 0.3 to 4 × 10^6 transformants/μg of ligated DNA to 1 to 5 × 10^8 transformants/μg of supercoiled plasmid. However, for the cI857 strain OR1265, rates as low as 2 × 10^4 transformants/μg of supercoiled plasmid were observed. Thus, plasmids containing the λ promoter P_L were first transformed into N99cI$^+$ (using the ligated DNA). OR1265 was then transformed with supercoiled DNA isolated from N99cI$^+$.) Transformed cells were selected by plating on L agar (L broth plus 1.5% Bacto-agar) containing 35 to 150 μg/ml ampicillin. The plasmid DNA from ampicillin-resistant colonies was isolated by a quick-boiling extraction technique (37) and screened for the presence of the insert by cleavage with appropriate restriction endonucleases and gel analysis of the DNA fragments.

Strategy for Construction of pLS501 and pLS502—An *Xba*I fragment of T4 dC-DNA (map units 22.37 to 17.90) was digested to completion with *Eco*RI and then partially digested with *Hin*dIII. The resulting products were ligated into *Eco*RI/*Hin*dIII cut pKG1810. This vector is a pBR322-based, amp^r, *galK* expression vector constructed by McKenney *et al.* (38) (see Fig. 1A). It contains a portion of the insertion sequence IS2, including a *rho*-dependent transcription termination site, downstream of the *Hin*dIII cloning site; the *E. coli galK* gene and pBR322 sequence follow the IS2 sequence. pLS501 and pLS502 contain 1090 bp (map units 21.15 to 20.06) and 550 bp (map units 21.15 to 20.60) of T4 DNA, respectively, cloned between the *Eco*RI to *Hin*dIII sites of pKG1810.

Strategy for Construction of pDH4112, pDH428, and pDH518—The plasmid pVH627 (gift of T. Mattson, University of Geneva) contains a 3400-bp *Eco*RI fragment of T4 dC-DNA (map units 24.3 to 21.15) cloned into the *Eco*RI site of pBR322 (see legend to Fig. 1 of Ref. 39). pVH627 was derived from pVH134 (34) which contains the same T4 *Eco*RI fragment cloned into pCR1. pDH4112 and pDH428 were constructed by ligation of this *Eco*RI fragment from pVH627 into *Eco*RI cut pLS502 and pLS501, respectively (Fig. 1A). To construct pDH518 (Fig. 1B), pDH428 was first digested with *Nar*I and *Bgl*II, and the 2620-bp fragment containing gene 41 was isolated. A *Nar*I to *Bam*HI fragment from pGC31 (gift of M. Cashel, National Institutes of Health) contains DNA identical to pDH428 from the *Nar*I site within the *galK* gene through the pBR322 sequence to the *Eco*RI site. However, between the *Eco*RI and the *Bam*HI sites of pGC31 lies 362 bp of DNA which includes the λ *Has*II fragment representing λ sequence 35819 to 35468 (40) and containing the λ promoter P_L and the λ *nutL* site. (An *Eco*RI site precedes the λ sequence and a *Bam*HI site follows.) Ligation of the fragments from pDH428 and pGC31 resulted in the plasmid pDH518.

Marker Rescue

The ability of T4 DNA cloned within a plasmid to rescue T4 amber mutations was tested by the liquid marker rescue technique described by Mattson *et al.* (34). DH1 (Su^+) cells containing various plasmids were grown to a density of 0.5 to 1 × 10^8/ml in FJ medium (41) and a 1-ml aliquot of cells was infected with 2 × 10^7 T4 amber phage. After standing at 37 °C for 8 min, the suspension was diluted 1/50 in FJ medium and incubated at 37 °C for 75 min with shaking. Chloroform was added to lyse the cells completely. The resultant phage were titered by plating on both Su^+ (CR63) cells and Su^- (ER21) cells. Marker rescue frequencies (Su^-/Su^+) (Table I) represent, then, the number of phage able to grow on the Su^- strain (presumably wild type phage) relative to the total phage burst.

41 Protein Assays

Complementation—Cell extracts were prepared from DH1 and OR1265 cells containing various plasmids and assayed for 41 protein activity in a gene 41 complementation assay as described (7), except

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that the host for the receptor extract was *E. coli* ER21, and reactions were incubated at 30 °C for 15 min. In this assay, a source of 41 protein and [^{3}H]dNTP (as well as the other dNTPs, rNTPs, and buffer) are added to a receptor extract made from T4 41$^-$ infected cells. The presence of 41 protein is observed as the incorporation of the [^{3}H]dNMP into acid-insoluble material. (Incorporation is reported as simply counts/min (Table II) since it is not possible to determine the amount of dNTP present in the receptor extract.)

Primer-dependent DNA Synthesis—As the 41 protein was purified from pDH518/OR1265 cells (see below), the 41 protein was also assayed by a RNA primer-dependent DNA synthesis assay (4). This assay measures the incorporation of [^{3}H]dTMP into DNA in a reaction mixture (10 μl) containing 41 protein, 25 mM Tris acetate, pH 7.5, 60 mM potassium acetate, 6 mM magnesium acetate, 10 mM dithiothreitol, 100 μg/ml bovine serum albumin, 0.5 mM ATP, 0.1 mM each of rCTP, rGTP, rUTP, dATP, dCTP, dGTP, and 0.1 mM [^{3}H]dTTP at 300 cpm/pmol, 230 pmol (as nucleotide) φX174 viral DNA, 40 μg/ml rifampicin, and the T4 proteins 43, 32, 44/62, 45, and 61 present at 2, 90, 40, 6, and 1.2 μg/ml, respectively. (The other T4 replication proteins were purified as described (42) except that 61 protein represents fraction V of the accompanying paper (25).) Under these conditions, the replication of the single-stranded DNA template is dependent on the synthesis of RNA primers catalyzed by T4 proteins 41 and 61. One unit of 41 protein is defined as the incorporation of 1 nmol of dTMP into acid-insoluble product (4) under these reaction conditions after 30 min at 30 °C.

Helicase Substrate and Assay—The hybrid, formed by annealing φX174 viral DNA to the heat-denatured *TaqI* band 6 (141-bp) fragment of φX174 replicative form DNA, was prepared and labeled at its 3′ terminus with [^{32}P]dGMP as described (10). Helicase reaction mixtures (10 μl) contained approximately 1 fmol of the φX174 *TaqI* band 6:φX174 viral DNA hybrid, 500 μM rATP, 200 μM each rCTP, rUTP, and rGTP, 60 mM potassium acetate, 25 mM Tris acetate, pH 7.5, 6 mM magnesium acetate, 5 mM dithiothreitol, 100 μg/ml bovine serum albumin, and 61 and 41 proteins as indicated. Our previous results (10) indicated that the 61 protein stimulation of the 41 helicase activity was increased by activation of 41 protein by incubation with rGTP (6, 42). Therefore, 41 protein (fraction V) at a concentration of 950 μg/ml was incubated for 3.5 min at 30 °C in 200 mM rGTP, 60 mM potassium acetate, 25 mM Tris acetate, pH 7.5, 6 mM magnesium acetate, and 5 mM dithiothreitol just prior to addition to the helicase assay. After 30 min at 37 °C, the reactions were stopped and the products were displayed on a 6% polyacrylamide gel as described (10).

Purification of 41 Protein from Cells Containing the Cloned Gene 41

A 50-liter fermentor culture of pDH518/OR1265 was grown at 29 °C in L broth plus 25 μg/ml ampicillin. At midlog phase of growth, the temperature was raised to 42 °C (to inactivate the cI857 repressor and thus induce expression from the promoter P_L cloned upstream of gene 41). Cells (200 g) were harvested after 2.5 h and frozen at −80 °C. Twenty-seven grams of cell paste were resuspended in 500 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 7.0, and 20% sucrose). A lysozyme solution (100 mg) (Worthington) in 25 ml of 100 mM Tris-HCl, pH 8.0, and 50 mM EDTA was then added, and the mixture was kept on ice for 45 min. The mixture was frozen in dry ice/ethanol and thawed in room temperature water 2 times. After the addition of 12 ml of 1 M $MgCl_2$, the solution was centrifuged 90 min at 40,000 rpm in a Beckman 45 Ti rotor. All subsequent steps were done at 4 °C. (The pH of buffers was measured at 25 °C.)

DEAE-cellulose Chromatography—A 430-ml column (3.3 × 50 cm) of DE23 (Whatman) was equilibrated in DE buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM $MgSO_4$, 25% glycerol, and 10 mM 2-mercaptoethanol). The 130,000 × *g* supernatant (520 ml) was diluted to 1,280 ml with DE buffer and loaded. After washing with 350 ml of DE buffer, the column was eluted with a linear gradient (4 liters) of DE buffer and DE buffer with 0.35 M KCl. Flow rate was 84 ml/h, and 21-ml fractions were collected. The peak of 41 protein activity eluted with 0.12 M KCl.

Single-stranded DNA-cellulose Chromatography—The DEAE-cellulose fraction (360 ml) was diluted 2-fold in buffer A (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 25% glycerol, and 10 mM 2-mercaptoethanol) and applied at 40 ml/h to a single-stranded calf thymus DNA-cellulose column (17.5 ml, 2.4 × 4.0 cm) equilibrated in AH buffer (buffer A plus 50 mM KCl), conditions under which 41 protein does not bind (10). The eluate coming through the column was collected.

Hydroxylapatite Chromatography—The DNA-cellulose eluate (760 ml) was applied to a Bio-Gel HT (Bio-Rad) column (240 ml, 9.5 × 25 cm) equilibrated in AH buffer. The column was washed with 250 ml of AH buffer and eluted with a linear gradient (4 liters) of 0 to 0.76 M $(NH_4)_2SO_4$ in AH buffer. Flow rate was 45 ml/h, and 22.5-ml fractions were collected. The peak of 41 protein activity eluted with 0.18 M $(NH_4)_2SO_4$.

Precipitation of 41 Protein—To an aliquot of the hydroxylapatite fraction (76 ml) was added an equal volume of 2.3 M (30%) $(NH_4)_2SO_4$ in AH buffer. After 24 h at 4 °C, a cloudy precipitate could be seen. The precipitate was collected by centrifugation at 20,000 × *g* for 30 min. The pellet was resuspended in 24 ml of AH buffer. After dialysis against buffer (10 mM Tris acetate, pH 7.5, 25 mM potassium acetate, 5 mM magnesium acetate, 25% glycerol, and 1 mM dithiothreitol), small aliquots were stored at −80 °C.

Other Methods

Protein concentrations were determined by the method of Lowry *et al.* (43) using bovine serum albumin as a standard. Discontinuous polyacrylamide gel electrophoresis of proteins was performed as described by Laemmli (44) with the following modifications. Running gel solution contained 10% acrylamide:*N,N′*-methylenebisacrylamide, (37.5:1), 0.188 M Tris-HCl, pH 8.8, 0.05% SDS, 0.03% TEMED, and 0.05% ammonium persulfate. Stacking gel solution contained 5% acrylamide (composition as above), 0.063 M Tris-HCl, pH 6.8, 0.05% SDS, 0.07% TEMED, and 0.1% ammonium persulfate. Gel loading solution (9% glycerol, 2% SDS, 57 mM Tris-HCl, pH 6.8, 4.8% 2-mercaptoethanol, and 0.05% bromphenol blue) was added to protein samples (at a ratio of ≥1:1) and the samples were incubated in a boiling water bath for 2 min prior to loading. Gels were run at 15 mA, constant current, in buffer containing 25 mM Tris, 0.19 M glycine, and 0.1% SDS. Gels were stained with Coomassie Blue as described (7).

The purified 41 protein (fraction V) was assayed for contaminating endonuclease activity as described (25) using either supercoiled M13 DNA and 1.0 μg of 41 protein or ^{32}P-labeled φX174 *TaqI* band 6 single-stranded DNA (heat-denatured) and 0.34 μg of 41 protein.

RESULTS

Cloning of the T4 Gene 41—Previous work by Mattson and colleagues (34) had assigned part of the T4 gene 41 to a 3400-bp *EcoRI* fragment (T4 map units 24.3 to 21.15). This assignment was based on the following marker rescue experiments. Infection by three different 41$^-$ amber mutant phage (N57, A461, and H601) of cells having a plasmid containing this *EcoRI* fragment resulted in wild type progeny at a high frequency. (The wild type phage arise presumably by recombination between the T4 DNA on the plasmid and the phage genome.) However, one T4 41$^-$ amber mutant tested, amN81, was not rescued, suggesting that only part of gene 41 was within this cloned T4 DNA.

To locate gene 41 more precisely, we constructed the plasmid pLS502 (Fig. 1A). This plasmid contains the 550-bp *EcoRI* to *HindIII* fragment of T4 DNA (map units 21.15 to 20.60) which lies immediately downstream of the 3400-bp *EcoRI* fragment. The T4 DNA was cloned into pKG1810, one of the pBR322-based *galK* expression vectors constructed by McKenney *et al.* (38) (Fig. 1A). As seen in Table I, the presence of pLS502 in the cell, but not the vector or a plasmid with the 3400-bp T4 insert, pVH627, results in a greatly increased marker rescue frequency for the T4 41$^-$ amN81. A control experiment using a T4 43$^-$ amber mutant shows no difference among pLS502, pVH627, and pKG1810. Thus, we assign the 41$^-$ amN81 mutation to the T4 *EcoRI* to *HindIII* fragment cloned in pLS502.

Taken together, these marker rescue experiments indicate that gene 41 crosses the *EcoRI* site at map unit 21.15. In an attempt to clone gene 41, we constructed a set of plasmids containing T4 DNA extending from this site (Fig. 1A). To test for the presence of 41 protein, extracts prepared from cells containing these various plasmids were compared with T4 gene 41 protein for the ability to stimulate DNA synthesis in a 41 complementation assay (7) (see "Experimental Pro-

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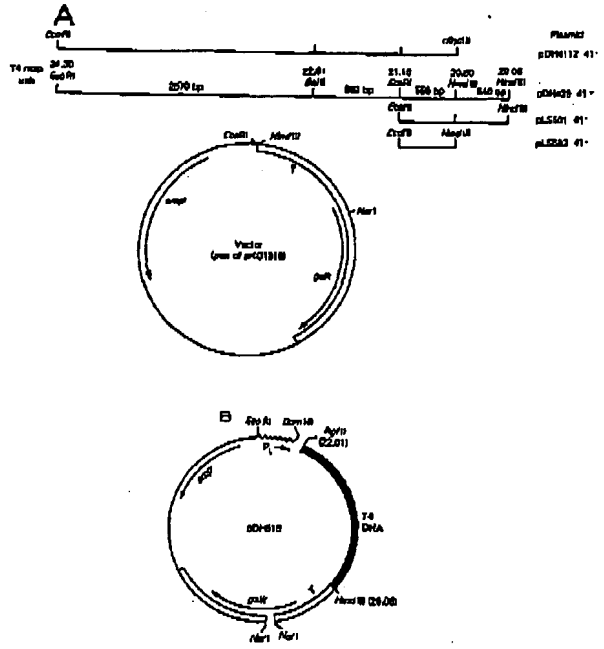


FIG. 1. **Plasmids containing the T4 DNA in the region of gene 41.** *A*, T4 DNA (*top lines*) representing the T4 physical map indicated (map units as given in Ref. 49) was cloned into the *EcoRI*/*HindIII* cut vector pKG1810 (38), containing the IS2 sequence 157 to 566 (50) which includes a transcription termination site (designated by T), the *E. coli* galactokinase (*galK*) gene, and pBR322 sequence with the ampicillin resistance gene (amp^r) (2068 to 4363 of the pBR322 DNA sequence) (51). Details of the plasmid constructions are given under "Experimental Procedures" and in the text. Restriction sites referred to in the text are indicated. The sites within the T4 DNA, previously mapped using T4 dC-DNA from phage (49), were confirmed by digestion of the plasmids. The results of assaying extracts of cells containing each plasmid for 41 protein complementation activity are indicated as 41$^-$ or 41$^+$ (see Table II). *B*, the plasmid pDH518. Immediately upstream of the T4 insert (map units 22.01 to 20.06) lies 363-bp of DNA which includes the λ sequence 35819 to 35468 (26) (shown here as ~~~). The λ DNA contains the leftward promoter of λ, P_L, followed by the antitermination site, *nutL*.

TABLE I

Marker rescue of T4 amN81 (41$^-$) by the plasmid pLS502

The strain DH1 (Su$^-$) containing the plasmids indicated was infected with either 41$^-$ (T4 amN81) or 43$^-$ (T4 am4335) as described under "Experimental Procedures." The production of wild type phage during the infection was then scored by the ability of the progeny phage to grow on ER21 (Su$^-$) and CR63 (Su$^+$) strains. Marker rescue frequencies represent the ratio of phage able to grow on the Su$^-$ ER21 strain (presumably wild type phage) relative to the total phage burst.

Strain	Marker rescue frequencies (Su$^-$/Su$^+$)	
	amN81 (gene 41)	am4385 (gene 43)
pKG1810/DH1 (no T4 DNA)	4.1×10^{-6}	2.6×10^{-6}
pLS502/DH1 (cloned T4 map units 21.15 to 20.60)	6.4×10^{-3}	1.3×10^{-6}
pVH627/DH1 (cloned T4 map units 24.3 to 21.15)	5.2×10^{-6}	1.3×10^{-6}

cedures," this paper). As seen in Table II, incorporation of [^{3}H]dAMP into DNA is observed with either authentic gene 41 protein or extracts from cells containing pDH428. This plasmid (Fig. 1*A*) contains 4500 bp of T4 DNA from map units 24.3 to 20.06. Since extracts from cells containing either

TABLE II

41 protein in plasmid containing cells measured by in vitro complementation

Extracts were prepared from the strains indicated, and 3 μl (Experiment I) or 5 μl (Experiment II) were assayed as described under "Experimental Procedures." See Fig. 1*A* for a description of each plasmid.

Experiment	41$^-$ recipient extract	Donor extract or 41 protein	[^{3}H]dAMP incorporated
			cpm
I	+	None	889
	+	Purified 41 protein[a]	7355
	+	DH1 (no plasmid)	821
	+	pLS501/DH1	837
	+	pDH428/DH1	7955
	−	pDH428/DH1	101
II	+	None	861
	+	Purified 41 protein[a]	5635
	+	pDH428/DH1	4832
	+	pLS502/DH1	710
	+	pDH4112/DH1	849

[a] 0.02 unit (complementation activity) of 41 protein (fraction III (7)).

pLS501 (cloned map units 21.15 to 20.06) or pDH4112 (cloned map units 24.30 to 20.60) do not complement the 41$^-$ extracts, we conclude that the T4 DNA spanning both the *EcoRI* site at 21.15 and the *HindIII* site at 20.60 is necessary for the production of active gene 41 protein.

We estimate the level of 41 protein complementation activity in the pDH428/DH1 extract to be at least as high as that observed after T4 infection. In order to produce higher levels of gene 41 protein, we constructed the plasmid pDH518, in which the T4 DNA from the *BglII* site (map unit 22.01) to the *HindIII* site (map unit 20.06) is now downstream of the strong λ promoter P_L (Fig. 1*B*). Such a construction places the T4 DNA in the same transcription direction as is observed for this region of the T4 genome (45, 46). The host for this plasmid is the strain OR1265 (27) which has the temperature-sensitive repressor cI857. Thus, at low temperature (29 °C), P_L is repressed. Shifting to high temperature (42 °C) inactivates repressor and results in expression from P_L. The results of such an experiment with pDH518/OR1265 can be seen by observing the Coomassie Blue-stained proteins separated on a polyacrylamide-SDS gel before and after the temperature shift (Fig. 2). One or 3 h after shifting to 42 °C, a new protein is observed which migrates with a molecular weight of 59,000 and co-migrates with 41 protein from T4-infected cells. Assaying the extracts from cells containing pDH518 indicates that, 3 h after the temperature shift, approximately 100-fold more gene 41 protein is produced than after T4 infection. The pDH518/OR1265 strain was then used to purify gene 41 protein.

Purification of 41 Protein—Table III and Fig. 3 summarize the purification of 41 protein from pDH518/OR1265 cells. While this purification is based on that used previously for T4-infected cells (7), several modifications have been made. First, we have increased by 10-fold the amount of lysis buffer used to initially resuspend the cells. Such an increase was made since we observed that a substantial fraction of 41 protein remains with the pellet after the 130,000 × *g* spin. The increase in the amount of lysis buffer decreases this loss, but we estimated that as much as ⅓ of the 41 protein still remains with the pellet. In addition, we have used a single-stranded DNA-cellulose column in the procedure to remove DNA binding proteins previously reported to contaminate 41 protein preparations (10). Third, we have used a hydroxyl-

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TABLE III

Purification of 41 protein from pDH518/OR1265 from 27 g of cells

Fraction	Volume	Protein	41 priming activity		
	ml	*mg*	*total units*	*units/mg protein*	*% recovery*
I. 130,000 × *g* supernatant	1280	4990	3.2×10^{6} [a]	64	100[a]
II. DEAE pool	360	970	1.7×10^{5} [b]	180	53
III. DNA cellulose eluate	760	1140	2.1×10^{5}	180	66
IV. Hydroxylapatite pool	250	450	1.4×10^{5}	310	44
V. Precipitated 41 protein	78	133	8.6×10^{4} [c]	650	27

[a] By gel analysis we estimate that approximately ⅓ of the 41 protein in the cell remains with the 130,000 × *g* pellet.

[b] The DE fraction represented approximately ⅝ of the total 41 protein eluted from the DEAE column.

[c] Calculated as if the entire hydroxylapatite fraction had been precipitated.

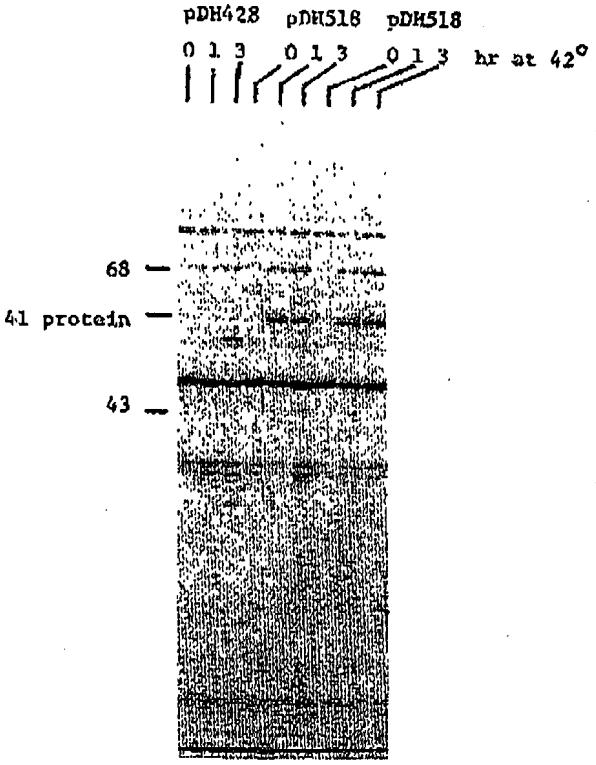


FIG. 2. Expression of gene 41 cloned downstream of the λ promoter P_L. 130,000 × *g* extracts of pDH428/OR1265 and two independent isolates of pDH518/OR1265 were prepared as described (7) immediately before and 1 or 3 h after the temperature shift to 42 °C. Proteins were separated on the 10% polyacrylamide-SDS gel shown and visualized by staining with Coomassie Blue. The positions of markers having molecular weights of 68,000 (bovine serum albumin) and 43,000 (ovalbumin), and the position of authentic gene 41 protein are denoted. (A band of 46,000 daltons, which we have identified as T4 *uvsX* protein, is not seen in these extracts of cells with pDH428 but is observed under other conditions of sample preparation (see "Discussion").)

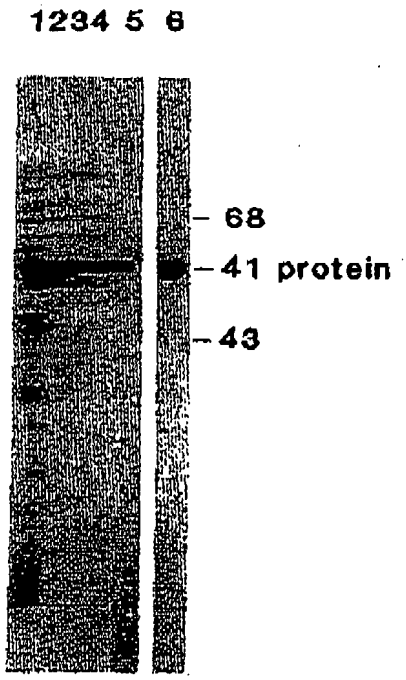


FIG. 3. 10% polyacrylamide-SDS gel showing the purification of the 41 protein expressed by pDH518/OR1265 cells. *Lane 1*, 5 μl, 130,000 × *g* extract (fraction I); *lane 2*, 3.5 μl of DE fraction (fraction II); *lane 3*, 7 μl of DNA-cellulose eluate (fraction III); *lane 4*, 4 μl of hydroxylapatite fraction (fraction IV); *lane 5*, 3 μl of redissolved, precipitated 41 protein (fraction V); and *lane 6*, 10 μl of redissolved, precipitated 41 protein. Positions of 41 protein purified from T4-infected cells and standards having molecular weights of 68,000 (bovine serum albumin) and 43,000 (ovalbumin) are indicated.

apatite buffer containing ammonium sulfate in place of the one containing potassium phosphate and $MgSO_4$ previously used for purifying 41 protein (7) since the use of the latter buffer resulted in a nonprotein precipitate in those fractions containing the peak of 41 protein. In addition, the ammonium sulfate buffer is convenient since we have determined that, by increasing the $(NH_4)_2SO_4$ concentration of the hydroxylapatite fraction to 1.24 M (16%), essentially pure 41 protein is precipitated from a solution at 4 °C, while many trace contaminants are not precipitated (Fig. 3). (This precipitation is temperature-dependent since no precipitation is observed if the solution is kept on ice.) Overall, our purification of the T4 41 protein from cells containing pDH518 yields 133 mg of homogenous enzyme from 27 g of cells. The purified enzyme is active in the RNA primer-dependent DNA synthesis assay using the seven purified T4 DNA replication proteins (Table III). As expected, this activity is dependent on the presence of the other priming protein, the T4 gene 61 protein (data not shown). The specific activity in this priming assay of the homogeneous 41 protein purified from cells with pDH518 is essentially the same as the activity per microgram of 41 protein (estimated from SDS gels) of the less purified hydroxylapatite fraction from infected cells (7).

The 41 protein from cells containing pDH518 also exhibits

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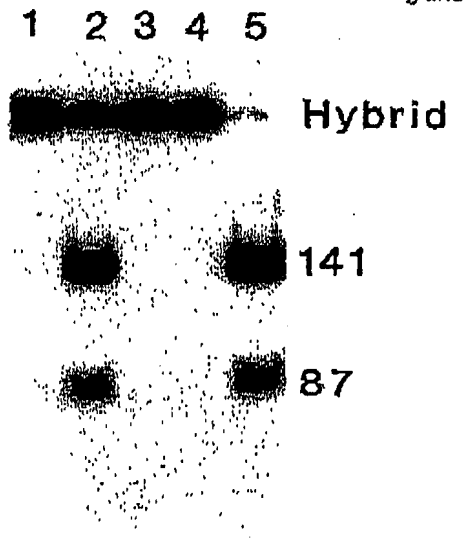


FIG. 4. DNA helicase activity of 41 protein purified from cells containing pDH518 is stimulated by 61 protein purified from a recombinant plasmid. Products of helicase reactions with the φX174 *TaqI* band 6:φX174 viral DNA hybrid, carried out as described under "Experimental Procedures," are displayed on a 6% polyacrylamide gel. *Lane 1*, 0.95 μg of 41 protein (fraction V); *lane 2*, 0.95 μg of 41 protein plus 0.024 μg of 61 protein (fraction V) (11); *lane 3*, 0.6 μg of 61 protein; *lane 4*, no enzyme; and *lane 5*, hybrid denatured by heating. The band at 87 bp is from the φX174 *TaqI* band 7 fragment contaminating the isolated *TaqI* band 6 fragment.

the helicase activity previously described for 41 protein from T4-infected cells (10) (Fig. 4). As seen previously, this activity is greatly stimulated by T4 61 priming protein. By counting the appropriate regions of the gel shown in Fig. 4, we estimate that 62% of the DNA hybrid was unwound with 41 protein in the presence of 61 protein, while 2.3% was unwound by 41 protein alone. Note that this reaction was carried out at 500 μM rATP, which is optimal for both DNA replication *in vitro* and 61 protein stimulation of the 41 helicase. The helicase activity of 41 protein alone is greater at 5 mM ATP (10).

DISCUSSION

Previously, studies of the bacteriophage T4 gene 41 protein have shown that this protein is a multifunctional member of the T4 DNA replication complex. It exhibits a single-stranded DNA-stimulated nucleotidase (7–9) and a DNA helicase activity (10), and, together with the T4 gene product 61, forms a primase which synthesizes pentaribonucleotides used to prime DNA replication on the lagging strand (1, 4–6). Previous attempts to discern the exact roles of 41 and 61 proteins within the primase have been hampered by the difficulty in purifying large amounts of these proteins from T4-infected cells. However, using proteins from T4 infections, it has been demonstrated that there is a stoichiometric relationship between the concentrations of 41 and 61 proteins required for primer synthesis (42) and that the helicase activity of 41 protein is stimulated by gene 61 protein and to a lesser degree by the polymerase accessory proteins (44/62 and 45) (10). This evidence suggests a direct interaction between 41 and 61 proteins and possibly the other replication proteins. We are interested in determining the nature of this interaction and thus have cloned the T4 gene 41 to obtain the large quantities of 41 protein we need to undertake further biochemical studies. We estimate that cells containing the plasmid with gene 41 downstream of the strong λ promoter P_L produce 100-fold more 41 protein than T4-infected cells. Our purification has yielded 133 mg of pure 41 protein from 27 g of such cells. The last step of the procedure, precipitation in 16% $(NH_4)_2SO_4$, results in a homogeneous protein of 59,000 daltons. Besides being visibly cleaner than any 41 protein we have previously purified from T4-infected cells, the protein from the clone is also free of any activities from the other T4 replication proteins and is free of endonuclease activity measured as described (25) using supercoiled plasmid or single-stranded DNA as templates. Our initial studies of the 41 protein (this paper) and the 61 protein (25) expressed by recombinant plasmids demonstrate that these proteins can substitute for those purified from T4-infected cells in the seven T4 protein DNA synthesis reaction that measures primer-dependent replication of DNA using a circular single-stranded template. Furthermore, the 41 protein from the clone also exhibits the helicase activity previously assigned to the T4 gene 41 product (10) and this activity is stimulated by the presence of the 61 protein (Fig. 4). In addition, our preliminary results[2] indicate that high concentrations of 61 protein alone can synthesize some oligonucleotide primers, but the priming ability of 61 protein under these conditions is greatly stimulated by 41 protein. Thus, within the T4 primase, 41 protein may primarily function as a DNA helicase, while 61 protein's primary role may be to catalyze primer formation. However, the stimulation of both helicase and priming activities when both proteins are present suggests that the primase complex catalyzes each activity more efficiently than the individual proteins.

Besides the above assays, the 41 protein from the clone is also active in the 41⁻ complementation assay. This assay measures stimulation of DNA synthesis by added 41 protein with the endogenous DNA in a crude extract of cells infected with a T4 mutant in gene 41 (7). However, we find that our purest fractions (IV and V) of 41 protein from the clone exhibit 2- to 5-fold less activity in this assay than expected, based on the amount of 41 protein added and on the primer synthesis activity of each of these fractions. At present, we do not know what reaction(s) of 41 protein are being measured in the complementation assay. Thus, it is not possible to determine whether this difference between the primer-dependent DNA synthesis assay using purified proteins and the complementation assay reflects an inherent difference between the 41 protein expressed by the plasmid and that from the T4-infected cell or is due to a contaminant in the latter preparation which also stimulates DNA synthesis in the crude extract. Better understanding of the functions of 41 protein in the replication complex should be useful in determining the reason for this difference.

Besides obtaining large quantities of purified 41 protein, our cloning has assigned the gene more precisely to the physical genome of T4. Our location of gene 41 between map units 22.01 and 20.06 is consistent with previous genetic mapping (47) and the marker rescue experiments of Mattson and colleagues (34). In addition, this region corresponds to an open reading frame predicted from the DNA sequence by Nakanishi and Alberts[3] (48) to express a protein of 54 kilodaltons. In the accompanying paper (25) we show that plasmids with T4 DNA from 18.68 to 17.32 map units express active gene 61 priming protein. We have also recently found that the plasmid pDH428 (Fig. 1A) contains the gene for the T4 *uvsX* recombination protein upstream from gene 41.[4]

[2] D. M. Hinton and N. G. Nossal, unpublished experiments.
[3] M. Nakanishi and B. Alberts, personal communication.
[4] D. M. Hinton and N. G. Nossal, manuscript in preparation.

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Thus, gene 41 lies between the *uvsX* gene and gene 61 in a region transcribed early after T4 infection. Our clones of gene 41 (and the surrounding DNA) should be useful in understanding the regulation of expression of this DNA replication protein in the T4-infected cell.

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